The impact of QMP on sexual fitness of male *Drosophila melanogaster* and the extent to which the effect of QMP is mediated by *FOXO* in the male *Drosophila* testes

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Abstract

Eusocial species exhibit high levels of complexity and a reproductive division of labour. One of the primary enforcers of reproductive division of labour in *Apis mellifera* is the production of Queen Mandibular Pheromone (QMP) by the queen. This reproductively restricts the female workers and excites the male drones to mate. Despite a divergence of ~330 million years, QMP also reproductively represses female *Drosophila melanogaster* and does not impact reproduction in males.

This study aimed to further analyse the degree of sex based differences in response to QMP in *Drosophila* and identify if low levels of FOXO in the testes mediate the reproductive resistance of males to QMP. We used feeding assays, performed with optical density and semi-quantitative analysis; fluorescence-based visualisation of insulin expression; mating assays and activity monitoring.

We found that QMP increases feeding in males during, but not following, exposure, possibly due to a full crop reducing the ability to further feed. QMP also reduces the activity of male *Drosophila.* Additionally, low *FOXO* does not seem to prevent the induction of starvation-like responses in the testes and finally, that the somatic support cells may allow sperm to develop insensitive to nutrition due to low *FOXO* levels.

The similarity of the sex based differences found between *A. mellifera* and *D. melanogaster* may indicate that the path of action of QMP acts through highly conserved signalling systems that differ by sex. This work contributes to the broader understanding of these social systems allowing for greater comprehension of one of the primary signalling systems in one of the most complex social species, contributing to the understanding of the factors that may have facilitated the development of such complexity.

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Abbreviations

9-HDA	(R)-9-hydroxydec-2-enoic acid
9-ODA	(E)-9-oxodec-2-enoic-acid
Akt	Protein kinase B
BAM	Bag of marbles promoter
CLM	Cumulative link model
DAM	Drosophila activity monitoring
DAPI	4',6-diamidino-2-phenylindole, a fluorescent dye that binds to DNA
DILPs	Drosophila insulin like proteins
F	Female
F1 generation	The first generation produced following a cross
F2 generation	The offspring of the F1 generation
FOXO	Forkhead box transcription factors
GAL4	A transcriptional activator that binds to UAS enhancer sequences
GFP	Green fluorescent protein
HOB	methyl p-hydroxybenzoate
HVA	4-hydroxy-3-methoxy-phenylethanol
InR	Insulin Receptor
LnK	A gene involved in insulin signalling encoding an adaptor protein
M	Male
MYA	Million Years ago
NOS	Nanos promoter
ns	Not statistically significant
PBS solution	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with .1% Tween-20
probe-seq	A cell type-specific bulk RNA sequencing method
PTx	Phosphate Buffer saline with 0.1% TritonX 100
QE	Queen equivalent
QL	Queenless
QMP	Queen Mandibular Pheromone
RDL	Reproductive division of labour
RNAi	RNA interference
RT-qPCR	Quantitative reverse transcription polymerase chain reaction

scRNA-seq	Single-cell RNA sequencing
TJ	Traffic jam promoter
Tukey HSD	Tukey's honestly significant difference test
UAS	Upstream activating sequence
UAS-FOXO	A transgenic construct that expresses FOXO under the control of UAS

Section 1 - General introduction

The chemical mediation of social behaviour

1.1 Eusociality

Eusociality is considered the most complex level of social organisation in the animal kingdom (Wilson and Sober, 1989). Eusocial species are defined by the reproductive division of labour, co-operative brood care and overlapping generations (Wilson, 1971).

Eusocial life history strategies can be found across diverse animal phyla, with examples found across mammalian and crustacean species. However, they are most frequently seen in insects (Wilson and Hölldobler, 2005), specifically within hymenoptera, in which eusociality has independently evolved at least nine times (Libbrecht and Keller, 2015). In some ecosystems, eusocial species account for more than half of insect biomass despite only representing around 2% of the total number of insect species (Bradshaw and McMahon, 2008), indicating the relative success of complex social structures associated with these species.

1.2 Reproductive division of labour

The reproductive division of labour, reproductive skew within a colony, is a key feature of eusociality which allows for caste-specific specialisation and increased efficiency of a colony. The level of reproductive skew found within eusocial species is often considered proportional to the level of eusociality exhibited by the species, but remains a subject of debate (Nonacs and Hager, 2011). For example, in some subspecies of the partially-social paper wasps (*Polistes),* if two females start a nest together, one will specify into a laying-dominant role and the other will specify into a worker-like role. The queen-like individual primarily engages in egg laying whereas the worker-like individual undertakes higher levels of non-laying activities such as bringing food, nest-building and leaving the nest. Once the first round of offspring hatch, the original workers are killed and the newly-hatched offspring become workers, retaining the original queen (Pardi, 1948). Despite this, it has been found in *Polistes carolina* that only an average of 60% of offspring within these colonies were produced by the dominant foundress with many subordinates producing the remaining offspring (Seppä et al., 2002).

In *A. mellifera*, a highly eusocial species, three types of bee are found within each colony: a queen, which is the primary reproductive organism of the hive; drones (males) whose sole purpose is to mate with virgin queens from other colonies; and workers who are responsible for all non-reproductive tasks within the hive. The sex of a bee is determined by its ploidy, with unfertilised haploid eggs developing into males and fertilised diploid eggs developing into females. With the exception of rare subspecies in which workers can reproduce parthenogenetically (Oldroyd et al., 2008), the queen will produce all of the female offspring in the colony and is also responsible for the overwhelming majority of drones produced.

The two types of female bee in *A. mellifera* are distinct castes. Caste specification is based on environmental factors, with royal jelly feeding throughout the developmental stages specifying between queens and workers. Worker bees of *A. mellifera* undertake a range of jobs. These are not consistent for a worker throughout its lifetime but rather are specified based on age-specific polyphenism, with workers tending towards jobs that grow in distance from the hive throughout their lives (Free, 1964; Johnson, 2003; Klowden and Palli, 2022; Seeley, 1982; Vance et al., 2009).

1.3 Reproductive constraints

The different levels of reproductive potential of individuals within a eusocial species must be attributed to the factors that these individuals are subjected to throughout their development and adult life. Any process that reduces the reproductive fitness of an individual relative to a queen is considered a reproductive constraint (Khila and Abouheif, 2010). A variety of reproductive constraints are found across many eusocial species including: the lack of ability to mate (e.g. *A.mellifera* workers (Koeniger et al., 2014a)), lack of spermatheca (e.g. *A.mellifera* workers (Koeniger et al., 2014b)), physical policing (e.g. *Bombus terrestris and A.mellifera* ((Lorenzo et al., 2012))), pheromonal control (e.g. *A.mellifera* (Winston and Slessor, 1998b)) and inherent sterility of castes (e.g. termite soldiers (Miura and Maekawa, 2020)).

The level of caste-specific reproductive repression is found to vary dramatically, even within eusocial species. In the Cape honeybee (*Apis mellifera capensis*), workers can reproduce parthenogenetically, producing clonal diploid offspring. The workers are equally genetically similar to these offspring as to those from queen-laid eggs. Therefore,

according to Hamilton's Law of inclusive fitness which dictates that social behaviour is based upon interplay between relatedness, cost and benefit, workers may be expected to police eggs laid by sister workers to a lesser extent than in a hive with non-parthenogenetically reproducing workers (Hamilton, 1963; Hamilton, 1964a; Hamilton, 1964b). Research into this found that the policing response of these colonies was colony-specific, with at least one colony not showing worker-egg policing (Beekman et al., 2002). The variance in the responses between colonies is thought to be due to varying degrees of hybridisation with *A. mellifera scutellata*, another subspecies of honeybee which does not reproduce parthenogenetically but which share a consistently defined geographic area of overlap between species locales (Hepburn et al., 1998). However, genetic analysis of the tested species would be required to test this hypothesis. There are also cases of eggs that are thought to avoid policing by mimicking queen egg-marking pheromones. For example, in anarchistic colonies, in which certain workers lay eggs even in the presence of a queen (Oldroyd et al., 1999), a much higher level of worker drone rearing is found than in non-anarchistic colonies. The transfer of eggs from anarchistic colonies into non-anarchistic colonies results in a lower level of policing than those produced by the workers of the colony itself. This finding was proposed by Oldroyd and Ratnieks (2000) to be due to the ability of worker anarchistic honeybees to produce a counterfeit queen-egg marking pheromone. Subsequent research attributed this phenomenon to the production of queen-like esters produced in the Dofour's gland, which coat the egg during laying, causing an increase in short-term persistence within the colony. However, it was noted that this could not be a true queen egg marking signal as it is generally not found on queen-laid eggs (Martin et al., 2004).

The communication of information between and within castes is important for well-functioning social colonies, with most communication orchestrated through chemical signalling (pheromones) (Grüter and Keller, 2016). As the primary reproductive organism in social species, it is especially important that a queen is able to exchange information with other castes. Queen pheromones are found across a range of social hymenoptera, typically in the form of simple cuticular hydrocarbons. In a comparative component analysis of queen produced worker-sterilising pheromones in a species of wasp (*Vespula vulgaris*), bee (*Bombus terrestris*) and ant (*Cataglyphis iberica*) by Van Oystaeyen et al. (2014), it was found that in all three tested species, repression was caused by long-chain linear and methyl-branched saturated hydrocarbons. The production of cuticular

hydrocarbons has been found to correlate to individuals' respective fertility in both the worker and queen castes in certain social insect species including the ant *Harpegnathos saltator* (Liebig et al., 2000). The similarity of these cuticular hydrocarbon-based signals on fertility in both queen and worker castes may suggest that queen pheromones of this type are modified fertility signals found in all pre-social ancestors of these species (Smith and Liebig, 2017).

In *A.mellifera*, queen mandibular pheromone (QMP) is more complex than other hymenopteran queen pheromones, comprising at least 9 components (Keeling et al., 2003), five of which are major components (Maisonnasse et al., 2010 ; Slessor et al., 1988) (Table 1), at least two of which (9-ODA and 9-HDA) are known to independently reproductively repress worker bees to the same level as QMP itself (Princen et al., 2019). QMP is measured in queen equivalents (QE), with one QE defined as the amount of queen substance produced by one queen in a single day. The complexity and level of redundancy found within the *Apis* genus is in stark contrast to that in the closest related genus, *Bombus*. For example, *Bombus impatiens* produces a simple cuticular hydrocarbon queen pheromone. The development of such a complex system in *A. mellifera* relative to other social species in the last ~81 million years of divergence, combined with the relatively wide range of species that it can repress, likely implies that this system co-opted an already established and highly conserved pathway.

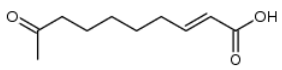
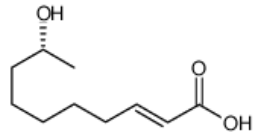
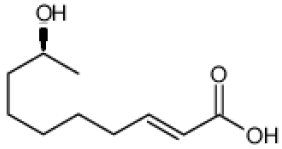
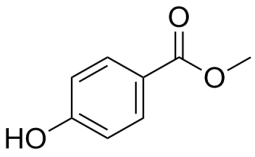
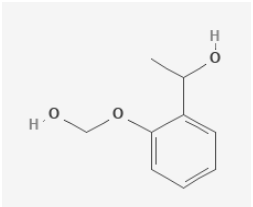
Name	Structure	Amount of independent reproductive repression of *A. mellifera* workers? (Princen et al., 2019)	Volume/QE (Pankiw et al., 1996)
(E)-9-oxodec-2-enoic-acid (9-ODA)		Independently reduces reproduction to the same extent as QMP	200µg
(R)-9-hydroxydec-2-enoic acid (9-HDA)		Independently reduces reproduction to the same extent as QMP	80µg
(S)-9-hydroxydec-2-enoic acid (9-HDA)			
methyl p-hydroxybenzoate (HOB)		Partially represses reproduction but not to the extent of QMP	20µg
4-hydroxy-3-methoxy-phenylethanol (HVA)		Partially represses reproduction but not to the extent of QMP	2µg

Table 1. **Five core components of *Apis mellifera* queen mandibular pheromone (QMP)** with structure, ability to completely reproductively repress and quantity found in one queen equivalent. Information on volume per queen equivalence taken from Alban Maisonnasse et al. (2010) reproductive repression data taken from Princen et al. (2019).

1.4 Queen Mandibular pheromone (QMP)

The theory of a honeybee queen pheromone dates back decades, with Butler (1954) publishing that the removal of queen substance triggers the production of queen cells proportionally to the lack of queen substance. He further theorised that the removal of the queen substance leads to the lifting of factors that inhibit “laying worker” development. This queen substance is now known to be the (*A. mellifera*) queen mandibular pheromone (QMP) (Naumann et al., 1991 ; Slessor et al., 1988), produced by the queen bee in the mandibular glands. However, since the discovery of QMP, it has also been found that queens with non-functional mandibular glands can reproductively repress workers indicating redundant repression methods, which may be indicative of other pheromones acting redundantly with QMP to repress fertility in workers (Maisonnasse et al., 2010). In a honeybee colony, QMP is considered to be a signal not only of the absolute presence of the queen, but also of the queen’s reproductive fitness, with high QMP levels thought to inhibit the rearing of new queens (Winston et al., 1989b; Pettis et al., 1995). The subject of how honestly QMP signalling reflects these factors has long been contested. One theory is that QMP production is proportional to reproductive fitness and as such acts as a real metric of the queen’s fitness (Keller and Nonacs, 1993), allowing for superseding to be established by the workers at a point where she is no longer considered to be fit enough (Butler, 1957 ; Seeley, 1985). Another is that QMP acts as a tool for the queen to to exercise repressive control over workers and only at a point in her lifespan when QMP production falls off can she be replaced (Winston and Slessor, 1992), as outlined by Maisonnasse et al. (2010). This remains a topic of debate.

1.5 The distribution of QMP

The distribution of QMP around a hive is thought to be through direct contact, with workers initially being exposed to it during retinue responses to the queen and subsequently distributing the QMP through direct contact with other workers and through the wax (Butler, 1954; Naumann et al., 1991). QMP is also commonly stated to be distributed through trophallaxis (Nixon and Ribbands, 1952). However, the extent to which this directly results in reproductive repression is unknown as it has been shown that injection of QMP does not directly inhibit worker ovary development (Verheijen-Voogd, 1959).

1.6 The effect of QMP on *A. mellifera.*

	Apis mellifera	*Drosophila melanogaster*
Female	Reproductive capacity - ↓ Retinue response - ↑ Feeding - ↑ Producing queen cells - ↑	Reproductive capacity - ↓ Retinue response - ? Feeding - ↑ Producing queen cells - NA
Male	Reproductive capacity - – Attraction to mate - ↑ Feeding - ?	Reproductive capacity - – Attraction to mate - ↑* Feeding - ?

Figure 1. **The effects of QMP on male and female *A. mellifera* and *D. melanogaster*** with increased (↑), decreased (↓) and unaffected factors (-) annotated. Factors that are not possible are annotated with NA and unknown factors annotated with a ?. *QMP exposed *D. melanogaster* show increased 'high intensity' courtship behaviour (Croft et al., 2017). *A. mellifera* female feeding information from personal communication (Anthony Bracuti - University of Leeds). All other references in text.

QMP has a number of effects on a colony (Figure 1), acting as both a releaser and primer pheromone, initiating immediate behavioural responses and long term physiological changes respectively (Mucignat-Caretta, 2014, Chapter. 5; Regnier and Law, 1968). As a releaser, it induces responses such as the attraction of drones, the coordination of swarming, and inducing the retinue response. As a primer, QMP exposure results in a reduction in the ovary activity of worker bees, reducing their reproductive fitness and increasing the proportion of worker bees within a population which have ovaries showing no sign of activation or the early stages of activation compared to non-exposed groups (Winston and Slessor, 1998a). In the event of a queen's death, workers are found to lay eggs which develop into drones at a much higher rate than seen in a queen-right colony. Workers can only produce drones as they lack a functional spermatheca and do not mate, meaning they can only produce haploid offspring. The production of drones in this setting

can be considered to be the best remaining option for a colony that can no longer produce diploid workers or queens to pass on its genetics, as the drones produced by the workers may still be able to mate with queens of other colonies on their mating flights, passing the genetics of the hive down to future generations (Beggs et al., 2007).

1.7 QMP response in *A. mellifera* workers

1.7.1 Sensing QMP (How is QMP detected)

It is currently hypothesised that QMP reception likely occurs through a combination of olfaction through olfactory receptors and/or gustation during trophallaxis which would be thought to occur through gustatory receptors (Naumann et al., 1991). Direct contact has been shown to be essential to the reproductive repression caused by QMP on worker honeybees (Lovegrove et al., 2020) with direct contact found to significantly decrease the number of mature oocytes found in comparison to indirect exposure, indicating either that olfaction alone is not essential in the reception of QMP, or that olfaction may only take place in very close proximity to the QMP.

1.7.2 Signal transmission

In order for the detected QMP to induce reproductive repression, the signal must be passed from the receptors to the reproductive tissues. The transmission of the QMP signal from the receptors is not fully understood. However several systems may mediate this process.

1.7.2.1 Biogenic amines (particularly dopamine) in response to QMP

Biogenic amines such as dopamine and octopamine are known to be differentially expressed in workers in association with queen exposure and have been proposed to mediate part of this QMP reduction of worker fertility (As summarised (Knapp, 2022, p.17)). A key component in this mediation is thought to be HVA (Table 2), a component of QMP that is structurally very similar to dopamine and is able to bind to dopamine receptors altering dopamine signalling (Beggs and Mercer, 2009). Workers with activated ovaries have been found to have increased brain dopamine (Harris and Woodring, 1995; Knapp, 2022), with queen-right colonies being found to have depressed dopamine in the brain compared to queen-less counterparts (Knapp, 2022). This was, however, not seen in

microcolonies exposed to QMP which nevertheless exhibited reproductive repression, potentially pointing to a combination of processes mediating this response. Factors proposed that were not accounted for in these experiments include the effects of brood pheromone, which has also been proposed to be a potential reason for the lower levels of reproductive repression found in hives than in experimental QMP only exposures (Knapp, 2022). It is however, also important to note, that non-hive based QMP experimentation relies on the use of synthetic QMP which typically comprises only a selection of components of QMP (typically 5) and, as such, would not be expected to induce any phenotypes that are caused by subcomponents, either directly or through interplay with other components.

The direct effect of dopamine administration has been found to differ between studies with Dombroski et al. (2003) finding increased ovary activation in queenless (QL) workers whereas Knapp (2022) found no differential activation. However, Knapp did find that inhibition of dopamine synthesis resulted in a reduction of ovary activation. The differences in the findings associated with dopamine administration have been proposed to be due to experimental differences, for example the use of Africanised versus European *A. mellifera* and blind versus non-blind ovary scoring (Knapp, 2022). The reduction of ovary activation in dopamine-synthesis inhibited worker bees infers that the modulation of dopamine levels may be a fundamental component of *A.mellifera* QMP reproductive repression; especially when it is taken into account that QMP is known to reduce worker brain dopamine levels. Whilst the Knapp dopamine administration results do not indicate that dopamine is completely proportional to ovary activity, this may be down to levels of redundancy (Knapp, 2022, p.68) with other factors such as brood pheromone potentially partially mediating this response.

There is evidence of Dopamine mediating sexual fitness outside of QMP exposure, with changes in dopamine signalling having been proposed to be important in the communication between the central nervous system and reproductive system in drones. This proposition is based on tissue-specific varying levels of dopamine receptor expression between the seminal vesicles, testees and mucus glands, with higher levels found in the seminal gland tissue (Matsushima et al., 2019). Similarly, in the queenless ant (*Diacamma sp.)*, dopamine signalling has been proposed to be responsible for differences in reproductive ability due to stimulation of the fat body (Okada et al., 2015).

1.7.2.2 Juvenile hormone and Ecdysone both change in queenless workers

The levels of both Juvenile hormone and Ecdysone are also found to change in queenless workers. These hormones are both known to be involved in the yolk deposition process in *Drosophila* and therefore could potentially mediate the repressive effect of QMP in *A. mellifera* (Meiselman et al., 2017). It is not currently known which of these pathways result in the signal transmission and it is possible that there is redundancy in the signal transmission pathway.

1.7.3 Effectors (How is the tissue altered)

1.7.3.1 Notch *E(spl)-C* genes

In *A. mellifera* workers, the development of the ovaries is restricted due to altered Notch signalling (Duncan et al., 2016). This is based on the finding that workers in queen-right conditions (QMP exposed), which are more likely to have repressed ovaries, express *E(spl)-C* genes (which are transcriptionally activated by Notch signalling) in the germarium of the ovaries. Furthermore, workers in the absence of QMP, which are more likely to have activated ovaries, are found to express these genes less. These genes are also found to be naturally low in queen bee ovaries, which are highly active. Therefore, the reduction of *E(spl)-C* gene expression in workers is considered to be inversely proportional to ovary activation. Additionally, inhibition of the Notch signalling pathway using a chemical inhibitor results in the activation of the ovaries of workers despite QMP exposure. These results indicate that low levels of Notch signalling in the germarium are associated with oogenesis (Duncan et al., 2016).

1.7.3.2 Programmed cell death

Cell apoptosis has also been proposed to be responsible for reproductive repression in workers (Ronai et al., 2016), with apoptosis at a mid-oogenesis checkpoint suggested to be responsible for the reduction in worker fertility in the presence of QMP. But there are some methodological considerations and further research in this area needs to be conducted to be able to determine the involvement in apoptosis in this response.

1.8 Non target species QMP responses

QMP has been documented to induce reproductive repression in several species, including in decapod crustaceans (Carlisle and Butler, 1956), the termite *Kalotermes flavicollis* (Sannasi and George, 1972), the house fly *Musca domestica L* (Nayar, 1963) and *Drosophila* (A. Sannasi, 1969 ; Camiletti et al., 2016 ; Lovegrove et al., 2019 ; Lovegrove et al., 2020 ; Lovegrove, 2020 ; Lovegrove et al., 2023). The consistency of the effect of *Apis mellifera* QMP on such a wide range of species is likely indicative of exploiting highly conserved systems and is in stark contrast to what is found in *Bombus impatiens* which does not induce reproductive constraint in *Drosophila melanogaster* despite a divergence of ~88MYA from *A. mellifera* (Lovegrove et al., 2019)

1.9 Why use *Drosophila*?

Drosophila is a well-established model organism, with documents of their use in biological research dating back to 1901 with the William Castle group at Harvard. Its low cost of maintenance, short generation time, availability for year-round experimentation and comprehensively understood genetics have made it an incredibly popular model organism to work with as summarised in Tolwinski (2017). It was also one of the first species to have its genome sequenced, facilitating functional genomics and leading to an influx of research in this species, including the development of a range of genetic techniques that are not established in *A. mellifera* (Adams et al., 2000).

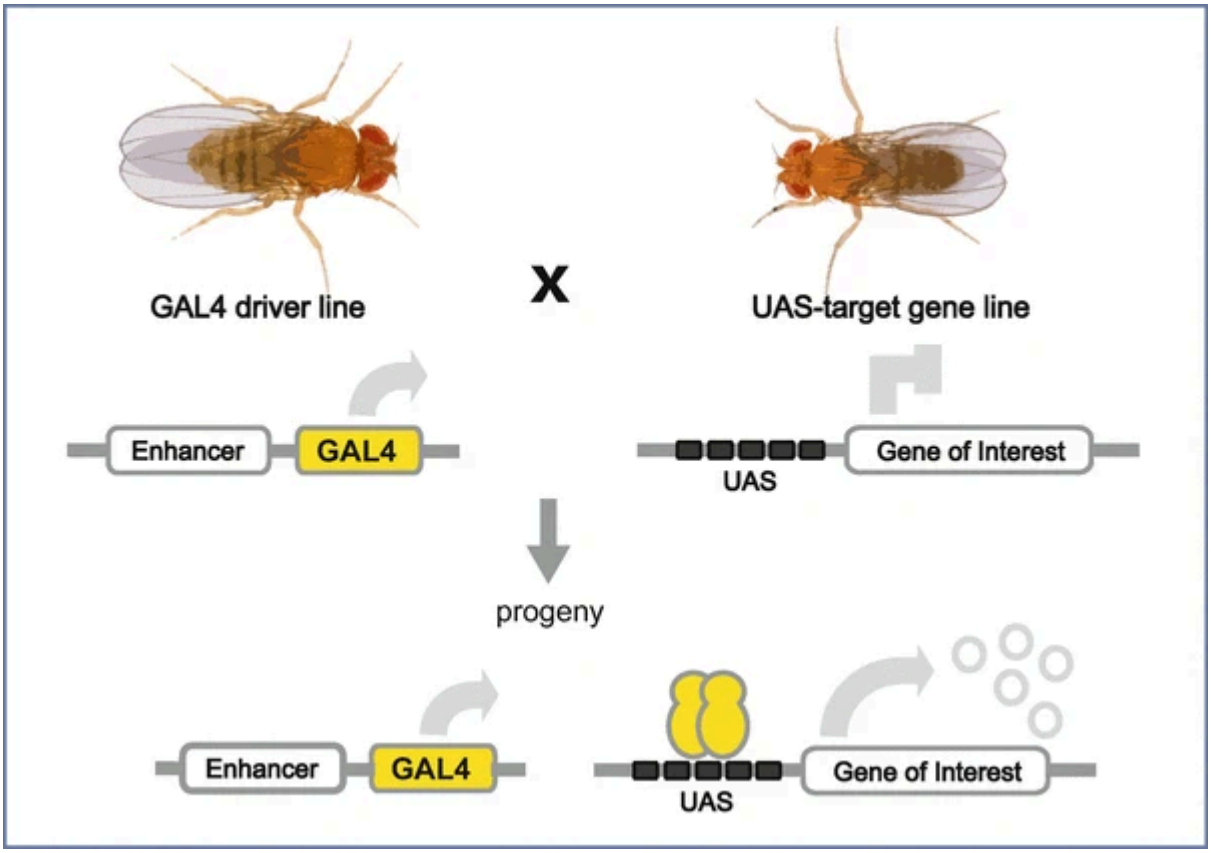


Figure 2. **Overview of the GAL4/UAS system in *Drosophila*.** This system relies on crossing a line with tissue specific GAL4 promotion with a line with tissues universally receptive to GAL4 resulting in expression of a gene of interest. This results in tissue specific expression of a gene of interest. Figure taken from Caygill and Brand (2016)

One such system is the GAL4/UAS system (Figure 2), a two line genetic approach which facilitates tissue-specific expression of genes of interest by crossing one line with a tissue-specific GAL4 (yeast transcription activator gene) gene with another line containing a UAS (Upstream activator sequence) connected to the gene of interest which will be expressed in all tissues that GAL4 is expressed in (Brand and Perrimon, 1993). This technique allows for tissue-specific over- and under-expression of a gene, allowing for analysis of its function within a system (eg. Tang et al. (2011)). Furthermore, the fact that QMP can induce similar responses in both honeybees and *Drosophila* in terms of repressing reproduction, suggests that a pathway that is conserved between the two species is likely being exploited by QMP (Lovegrove et al., 2023). The higher level genetic analysis that can be done with *Drosophila,* combined with the more comprehensive understanding of the organism, means that it may be easier to establish an action pathway of QMP in this species which may then allow for better understanding of the pathway taken by QMP in honeybees.

1.10 Effect of QMP on female *D. melanogaster*

QMP exposure has been found to reproductively repress *female D. melanogaster*, with a reduction found in the number of eggs and total size of the ovaries (Camiletti et al., 2013; Lovegrove et al., 2019), despite an evolutionary divergence of ~330 million years from *A. mellifera* (Misof et al., 2014). Significantly reduced mature egg numbers were found at QMP exposures of ≥ 5.7QE and significantly reduced ovary area was found at levels of ≥ 11.4QE. (Camiletti et al., 2013)(QE values adjusted as specified by Lovegrove (2020)). Camiletti also found a reduction of pupae production and offspring eclosure following QMP exposure but only at 5.7QE, likely indicating that that the induced effect only occurs within a threshold of exposure higher than 2.9QE and lower than 11.4QE. Further research by Lovegrove et al. (2019) found a reduction in the number of mature oocytes in *D. melanogaster* at QMP QE values ranging from 3.25-26 (Figure 3), concentrations much higher than is used on honeybees.

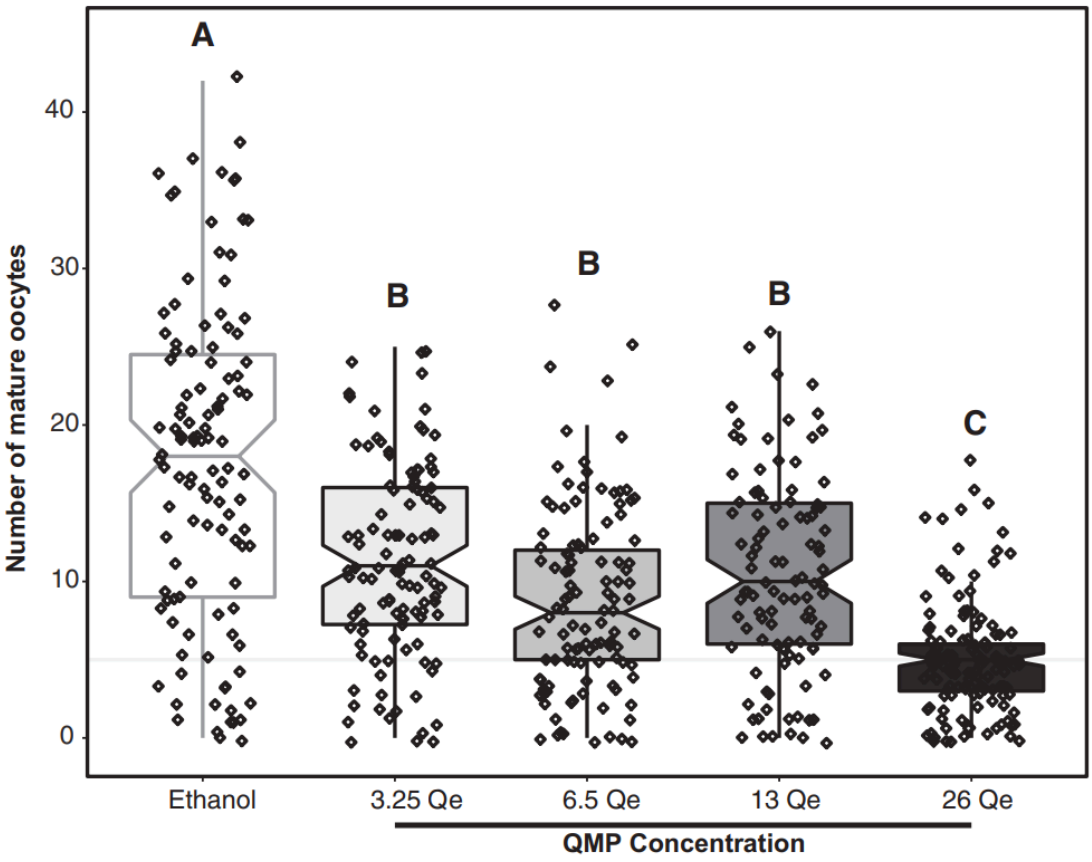


Figure 3. **The effect of QMP on fertility of female *Drosophila melanogaster*** at a range queen equivalent exposures of QMP (dissolved in ethanol) from 0 to 26. Groups that do not share a letter are considered significantly different. Graph taken from (Lovegrove et al., 2019)

Detection of QMP by female *Drosophila*, as in *A.mellifera* is not comprehensively understood. However, direct contact has been shown to impact the level of QMP response at QMP QE levels of both 13 and 20, with mean egg number and mean ovary area significantly lower in groups with direct contact than those with limited access (Camiletti et al., 2016). Camiletti et al. (2016) also used loss of function mutants for the olfactory co-receptor Orco, which is known to facilitate all odorant receptors, in order to establish whether olfaction is essential in the sensing of QMP, finding QMP not to impact ovary size of these mutants. RNAi was also used to knockdown a range of olfactory receptors, with the finding that knockdown of Or49b, Or56a and Or98a resulted in significant reduction in the impact of QMP on ovary size and egg number (Camiletti et al., 2016). These findings suggest that odorant reception (olfaction) is essential for the action of QMP in *D.*

melanogaster. The Orco mutants do, however, have a smaller ovary size than the wildtype controls and therefore may not be expected to reduce in size even with QMP exposure, given that there may be a maximum size reduction that can occur in these tissues. It has also been found that removal of the olfactory organs, the antennae and maxillary palps cause intermediate QMP responses (Lovegrove et al., 2020). These results suggest that either olfaction helps facilitate the QMP response without being essential, indicating levels of redundancy in the system, or that olfaction of QMP can occur in organs other than the antennae and the maxillary palps such as the body or legs.

Unlike in *A.mellifera*, QMP does not seem to act through Notch signalling in the *D. melanogaster* ovary, with QMP-exposed versus control *Drosophila* females being found to have no difference in the expression of the *E(spl)-C* genes that are affected by QMP exposure in the ovaries of honeybee workers (Lovegrove, 2020). This is not surprising as Notch signalling in *Drosophila* promotes oogenesis and so would not result in reproductive repression as is seen in *A. mellifera*. Whilst this does show that Notch signalling is not impacted in the ovaries of QMP-exposed female *Drosophila*, it does not necessarily mean that it is not occurring elsewhere in the body, as QMP is hypothesised to be acting through highly conserved pathways and impacting Notch signalling in *A. mellifera*.

The reproductive repression of female *Drosophila* in response to QMP has been shown to repress signalling through the insulin signalling pathway, inducing a starvation effect, with QMP-exposed individuals eating significantly more than those that are non-exposed. It was also shown that the gluconeogenesis-associated genes *Phosphoenolpyruvate carboxykinase 1* and *Fructose-1,6-bisphosphatase* did not change in QMP-exposed individuals as is seen in starving controls (Lovegrove et al., 2023). Together, these results suggest that QMP induces a starvation response without directly inducing starving in female *Drosophila*.

1.11 Insulin signalling system overview

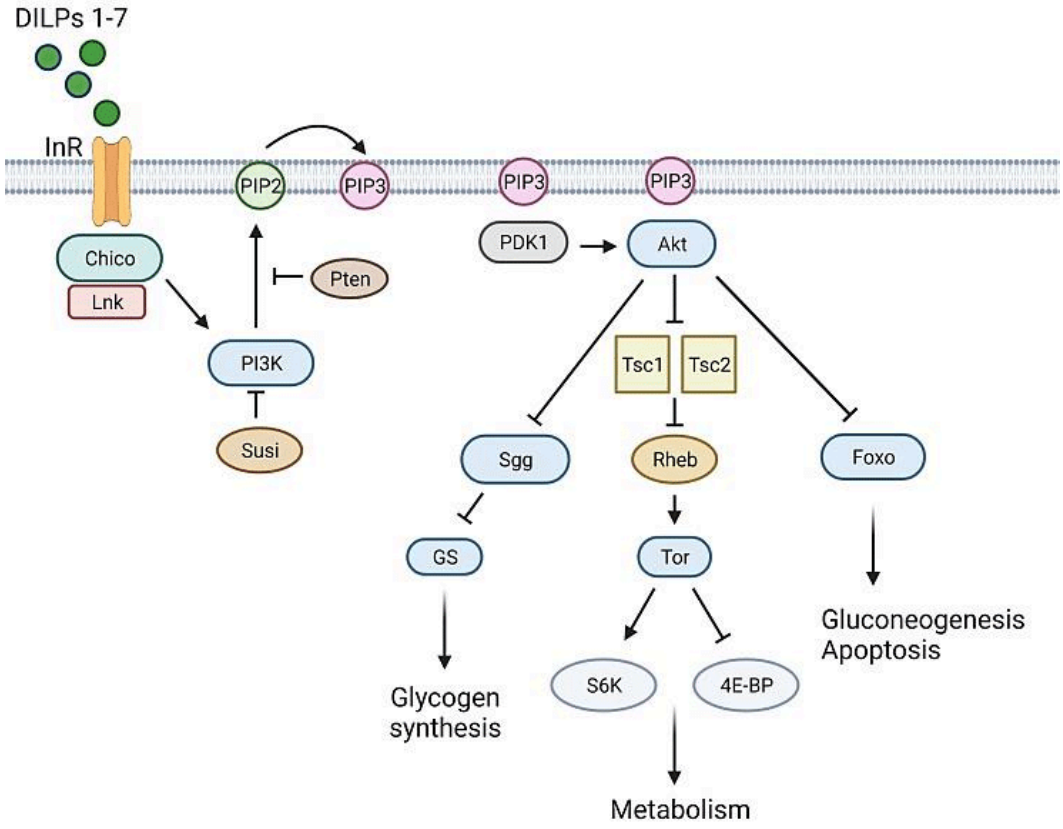


Figure 4: **Overview of the Insulin signalling system.**
Drosophila insulin-like peptides (DIPLS), bind to InR (the insulin receptor) and activate the insulin signalling pathway. Consequently, InR undergoes a conformational change exposing its juxtamembrane domains. Chico then interacts with the exposed juxtamembrane domains to form a complex which is stabilised by the recruitment of other molecules such as LnK. LnK is a tyrosine kinase that binds to and stabilizes the InR-Chico complex. It then autophosphorylates, phosphorylating its own tyrosine residues. This allows for Lnk to phosphorylate the InR tyrosine residues, triggering the signalling cascade. PI3K, a kinase, then catalyses the phosphorylation of the cell membrane phospholipid PIP2 to generate PIP3. PI3K activity can be interrupted by Susi and Pten which are phosphatases that dephosphorylate PIP3, which is a second messenger molecule produced by PI3K. This disrupts the signalling pathway. Susi is constitutively active and Pten operates with regulated activity. PIP3, generated by PI3K activates PDK1, a serine/threonine kinase, activated by which then phosphorylates Akt contributing to the activation of Akt which is also a serine/threonine kinase. Activated Akt acts in three major pathways.

Firstly, Akt phosphorylates Sgg a serine/threonine kinase, which in its active form phosphorylates glycogen synthase which restricts the ability of GS to synthesise Glycogen. Phosphorylated Sgg results in reduced repression of GS by Sgg, meaning that GS is no longer inhibited by Sgg. Activated GS, glycogen synthase, catalyses the conversion of glucose 1-phosphate to glycogen which acts as energy storage in the cells. Secondly Akt interacts with the Tsc1 Tsc2 complex, Tsc1 is a tuberin is a tumor suppressor protein that forms a complex with Tsc2 which is also a tumor suppressor protein. Activated Akt phosphorylates Tsc2 weakening the Tsc1-Tsc2 interaction. causing Tsc1 to dissociate from tsc2. Free Tsc1 facilitates the exchange of GDP (Inactive) to GTP (Active) on Rheb. This then facilitates the binding of Rheb-GTP to mTORC1 (TOR (not Tor)). 4E-BP which Is a regulator of translation, phosphorylated by mTORC1 consequently altering it's activation state. Finally, Akt also represses FOXO which are forkhead transcription factors which regulate downstream effects of insulin signalling by regulating gene expression.

Graph taken from Liguori et al. (2021). Information from Laplante and Sabatini, (2009); Laplante and Sabatini, (2009); Das and Arur, (2017); Saxton and Sabatini, (2017); Biglou et al., (2021); Liguori et al. (2021).

The insulin signalling pathway is highly conserved across the animal kingdom and is essential to many fundamental elements of animal biology including development, behaviour, and metabolism (Biglou et al., 2021). Due to the highly conserved nature of the system, the *Drosophila* insulin signalling system (ISS) is highly representative of the system across the animal kingdom (Das and Arur, 2017) (Figure 4).

D. melanogaster has eight insulin-like peptides,but this number varies in other species (Biglou et al., 2021). The insulin signalling pathway is activated at a cellular level when *Drosophila* insulin-like proteins (DILPs) bind to InR, the insulin receptor, resulting in a structural change in the receptor's conformational region, exposing its juxtamembrane domains (Tatar, 2021). Chico interacts with the exposed juxtamembrane domains to form a complex which is stabilised by the recruitment of other molecules such as LnK, an adaptor protein that binds to and stabilises the InR-Chico complex (Tatar, 2021). This results in the activation of phosphoinositide 3-Kinase (PI3K) which catalyses the phosphorylation of the membrane bound protein PIP2, generating PIP3 (Levina et al., 2022)(Summarised by Liguori et al. (2021)). PIP3, the secondary messenger molecule which is synthesised by the addition of a phosphate group to PIP2, activates both PDK1 and Akt (Biglou et al., 2021), which are both serine / threonine kinases. PDK1 also phosphorylates Akt, which is essential for its full activation (Levina et al., 2022).

The signal cascade splits into three action paths at this stage. Along the first path, Akt inactivates Sgg by phosphorylating it (Papadopoulou et al., 2004). As a result, Sgg is less able to phosphorylate glycogen synthase leading to an increase in activated Glycogen synthase (GS) (Papadopoulou et al., 2004) resulting in increased Glycogen synthesis, the energy storage molecule of cells. In the second path, Akt represses two tumour suppressor proteins, tuberous sclerosis complex 1 and 2 (Tsc1/Tsc2) (Das and Arur, 2017). This causes the complex formed between the two molecules to weaken, causing dissociation between the molecules (Das and Arur, 2017) resulting in the activation of Rheb, this facilitates the binding of Rheb-GTP to mTORC1 (Saxton and Sabatini, 2017). mTORC1 impacts protein synthesis (Saxton and Sabatini, 2017), lipid synthesis (Laplante and Sabatini, 2009) and cell growth (Saxton and Sabatini, 2017). In the third pathway, Akt adds a phosphate group to Forkhead box transcription factors (FOXO) (Biglou et al., 2021). This restricts FOXO from entering the nucleus. As FOXO is a transcription factor that acts within the nucleus, it then can not regulate the expression of genes. FOXO activity is known to impact gluconeogenesis and cell apoptosis (Biglou et al., 2021).

FOXO expression level is also important in tissue-specific nutritional developmental sensitivity, with FOXO in a tissue known to mediate the level of phenotypic plasticity (Tang et al., 2011).

1.12 Digestive system overview in *D. melanogaster*

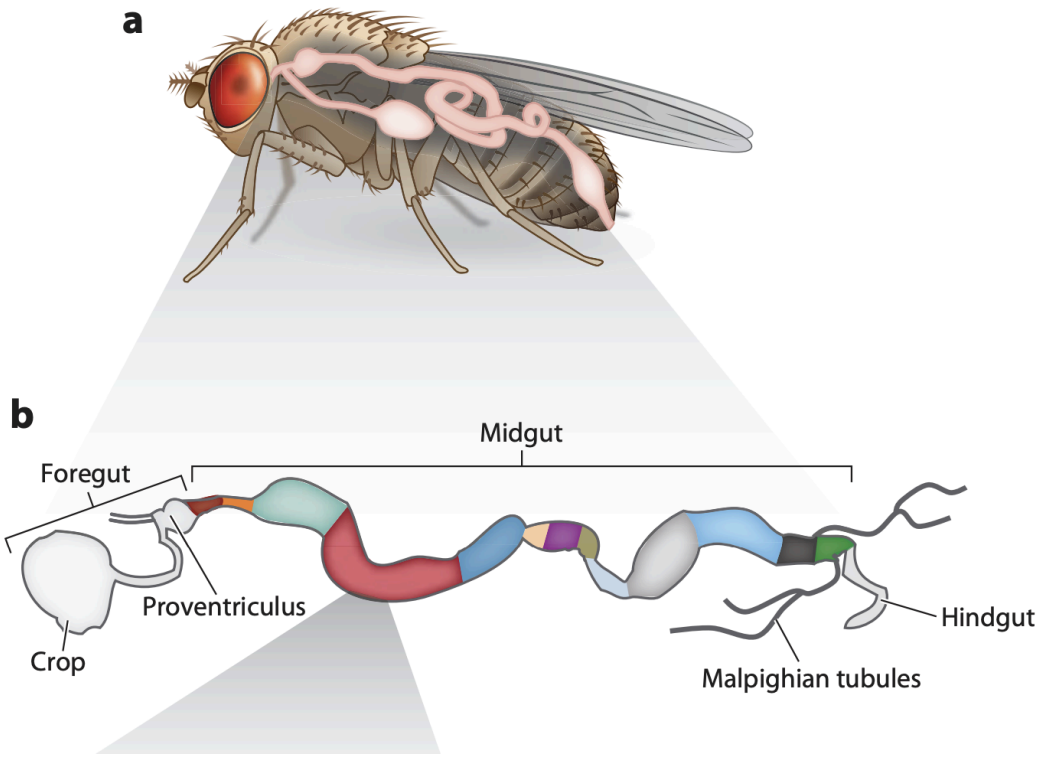


Figure 5. **Digestive system of *Drosophila***. A. Overlaid over the body of the an adult. B. isolated with organs specified. Taken from (Lemaitre and Miguel-Aliaga, 2013).

The digestive tract in *Drosophila* (Figure 5) comprises three regions: the foregut, midgut and hindgut.

Adult *Drosophila* feed using their proboscis to ingest liquid. Following ingestion the ingested food is transported into the foregut. The foregut consists of the pharynx, crop, esophagus, and proventriculus (the foregut section of the cardia). Food entering the foregut moves into the esophagus and crop duct. From the esophagus, food is transported into the proventriculus, which regulates the transfer of the food into the midgut. Before the food is transported into the midgut, it is exposed to the secretions of

the salivary gland and may be redirected into the crop (Zhu et al., 2024). The crop is an organ that is similar in function to the mammalian stomach in that it stores food for pre-processing before it is transported to the midgut (Cai et al., 2020; Zhu et al., 2024).

The midgut of *Drosophila* functions similarly to the small intestine in humans. It is a long tube lined with digestive and hormone producing cells. It contains at least ten different regions, each with distinct structures that facilitate different stages of the digestive process (Marianes and Spradling, 2013). One notable region within the midgut is the copper cell region, a highly acidic section that, like the crop, is considered to perform a stomach-like function in *Drosophila* (Cai et al., 2020 ; Lemaitre and Miguel-Aliaga, 2013 ; Strand and Micchelli, 2011).

The *Drosophila* hindgut processes food after its progression through the midgut. It consists of three major components; the pylorus, ileum and rectum (Fox and Spradling, 2009). The pylorus is a value-like structure that immediately follows the midgut and controls flow of the food from the midgut to the hindgut. The ileum follows, and it mediates the absorption of water (Takashima et al., 2008). The remaining gut contents, including excretory products removed from the hemolymph via the malpighian tubes, are expelled into the hind gut, then progress to the rectum (Denholm and Skaer, 2005). This also acts as a site of water absorption from the contents of the gut (Miguel-Aliaga et al., 2018). The remaining gut contents then pass through the rectum, to be released as waste.

1.13 The effect of QMP on males

1.13.1 *A. Mellifera*

A. mellifera mating occurs aerially in drone congregation areas, where large numbers of drones congregate. Virgin queens fly though drone congregation areas, where they mate with multiple drones before returning to their colony. In order for this to occur, drones must be able to locate a congregation site and find the queen as she passes through (Galindo-Cardona et al., 2012).

A component of QMP is known to act as a mating pheromone. 9-ODA has been found to attract drones towards queens (Brandstaetter et al., 2014 ; Gary, 1962; Gary and Marston, 1971). However, a synthetic blend of QMP has been found not to attract drones. Despite

being considered a mating pheromone, QMP is not likely to specify specific drone congregation areas as the queens enter them after the drones have congregated, as summarised in Koeniger and Koeniger (2004).

The difference in behaviour between sexes in response to QMP exposure indicates a sexually dimorphic response. This is interesting, given that QMP is hypothesised to act through highly conserved pathways, and it indicates that the pathways might be inherently different based upon sex.

Very little is known about the effect of QMP on cellular signalling in drones. This is probably due to the fact that drone fertility doesn't obviously vary between queen-right and queenless hives. It is, however, important to note that this does not mean that it is not affected by QMP. Another factor which likely plays into the lack of research in this area is the relative difficulty of performing fertility analysis on males compared to females. Typically, workers are assessed for fertility by analysing the number of mature oocytes found within their ovaries, whereas males are assessed based on the average offspring produced after a mating event or based on sperm density, motility analysis and genotyping of offspring (Metz and Tarpy, 2019 ; Rangel and Fisher, 2019 ; Yániz et al., 2020). These techniques are more demanding to conduct, especially if analysis is done post-mating as *A. mellifera* bees mate in pre-defined drone congregation areas with large competition between males. As a result, *A. mellifera* drone fertility research, based on offspring production, would therefore probably rely on artificial insemination methodology for reliable tracing of paternal lineage, which is a highly intensive process (Khan et al., 2022).

1.13.2 *D. melanogaster*

Little research has been conducted on the effect of QMP exposure on male *Drosophila*. Therefore, the extent to which they mimic the responses seen in QMP-exposed drones is not comprehensively understood. However, male *D. melanogaster* have been found to consistently orient towards QMP in a T-maze chamber (Croft et al., 2017). This effect is similar to that seen in drones, with drones known to be attracted to mate with virgin queens by QMP (Brandstaetter et al., 2014). Specifically, this effect has been attributed to 9-ODA (Butler, 1971; Gary and Marston, 1971; Wanner et al., 2007). Furthermore, it has been found that QMP affects the distribution of time in that male *Drosophila* spend in various stages of courtship when attempting to mate, with a higher proportion of the time spent at high courtship intensities (licking, attempting copulation and copulation) (Croft et al., 2017). This indicates that QMP may also act as a sex pheromone in *Drosophila*.

Research from the Duncan Lab (Chambers, 2023) found that QMP exposure in male *Drosophila* had no impact on average offspring count or average copulation length. The fertility findings contrast with those for female *Drosophila,* which have reduced fertility when exposed to QMP.

It was also noted that, unlike with female *Drosophila,* feeding behaviour differed between QMP-exposed and starved males, with QMP-exposed individuals having a significantly reduced average length of time before copulation, which was anecdotally attributed to reduced feeding activity before mating behaviours in comparison to starved controls. This is potentially indicative of differential levels of starvation-induced between the sexes, not only reflecting sex-based dichotomy as is found in *A. mellifera*, but also potentially suggesting a sex-based difference in the insulin signalling response between male and female *Drosophila*.

True starvation has been shown to reduce insulin signalling in male *Drosophila* (Sudhakar et al., 2020). However, the genitals of *Drosophila* are also known to be insulin insensitive (Tang et al., 2011). This results in tissue-specific growth during development that is not proportional to nutritional intake, with male testes found to be very similar in size between individuals regardless of body size (Shingleton et al., 2009). This has been attributed to the lower FOXO levels found in the male testes compared to other more nutritionally plastic tissues, with expression of FOXO in the genital imaginal disk found to render the

tissue nutritionally sensitive, reducing the size it grows to during development (Tang et al., 2011). Artificially increasing *FOXO* levels (GAL4/UAS) in the genital arch of *Drosophila* males also restores nutritional plasticity in genital tissue development, indicating that development can be impacted by insulin signaling post-tissue specification (Dreyer and Shingleton, 2019). This was found to negatively impact a large range of sexual fitness metrics, with male *Drosophila* with genital *FOXO* expression found to be less likely to copulate with a female, less likely to produce offspring from copulation and likely to produce less offspring than non *FOXO* lines (Dreyer and Shingleton, 2019). This indicates that the genitals of male *Drosophila* are insulin resistant and therefore have limited susceptibility to reduced sexual fitness due to underdevelopment. Given that the effect of reproductive repression in female *Drosophila* is thought to be mediated at the level of the genitals through insulin signalling, it is possible that this is the reason for the dichotomy between the sexes in response to QMP. However, further research would be needed in order to confirm this.

The results of these experiments, when considered alongside the known sex-based differences in QMP fertility in *A. mellifera,* indicate a sex-based dichotomy to QMP that is conserved across species, potentially indicating that the difference is caused by highly conserved pathways that differ by sex. Understanding the differences in the male and female response could shed light on the mechanistic pathways of the QMP which would allow for greater understanding of pheromonal communication in social insects and potentially allow for better understanding of how highly complex eusocial species such as honeybees evolved.

1.14 Objectives and aims

1. **Does QMP exposure affect feeding behaviour in male *Drosophila*?**

In order to identify the magnitude of the feeding impact of QMP on male *Drosophila*, a quantitative feeding assay was used, as used by Lovegrove et al. (2023). This was used to identify whether a starvation response was induced in males and if so, to what extent this response was consistent with female *Drosophila* (Lovegrove et al., 2023).

2. **Is *FOXO* responsible for the lack of effect of QMP on male *Drosophila* genital tissues**

If an insulin signalling response is being induced but not causing fertility differences, this may be down to a lack of insulin sensitivity in the genital tissues. This was tested through the use of GAL4/UAS lines in order to upregulate *FOXO* in the genital tissues, rendering the tissue development insulin signalling dependent (Dreyer and Shingleton, 2019 ; Tang et al., 2011). Should QMP cause decreased sexual fitness in these crosses, it is likely that the dichotomy between male and female *Drosophila* QMP responses is due to a lack of reception of the induced insulin signal in the genitals in the males that is not seen in the females.

1.15 Importance

The results of these experiments will help in understanding the similarities and differences found between male and female *Drosophila* and honeybee responses to QMP, improving our overall understanding of the impact of QMP on reproduction. This should contribute to the understanding of the pheromonal evolution that allowed for one of the major transitions in social evolution.

Furthermore, whilst *A. mellifera* are a farmed species and therefore are considered to be largely protected from many of the challenges facing wild species, this is not true for all subspecies. The understanding of the systems within species allows for more tailored conservation efforts in these subspecies and the potential to counter problems proactively.

Finally, an enhanced grasp of animal systems may contribute to a greater understanding of many conserved systems, such as the insulin signalling pathway which has the potential to prove useful for medicine.

Section 2 - General Methods

2.1 *Drosophila stocks and maintenance*

D. melanogaster stocks were maintained at 25°C with a 12-hour light-dark cycle (9am/9pm). Stocks and crosses were maintained on modified Bloomington media comprising; 27 g agar, 200 g cornmeal, 140 g sugar, 50 g yeast, 20 mL propanoic acid (0.67% concentration in final solution assuming no water loss during cooking), 20 mL nipagen and 3 L distilled water.

Crosses were established using male GAL4 driver lines and female UAS lines (Table 2), with a larger number of females than males at ~5F/3M per tube.

Name	Stock centre	ID	Expression	Genotype
Oregon-R	Bloomington	25211	Wildtype	Oregon-R-modENCODE
UAS-FOXO	Bloomington	9575	FOXO expression in areas of GAL4 expression	y[1] w[*]; P{w[+mC]=UAS-foxo.P}2
TGPH4	Bloomington	8164	Expresses a fusion protein comprising a PIP3 localiser and GFP	w[118]; P{w[+mC]=tGPH}4
NOS-GAL4	Bloomington	64277	Strong GAL4 expressed in germ line cells	y[1] w[*]/Dp(1;Y)Bar[S]Yy[+]; P{w[+mC]=GAL4::VP16-nanos.UTR}1C
BAM-GAL4	Aspden Lab	NA	GAL4 expression in late spermatogonia and early spermatocytes	bam-GAL4 VP homozygous on III
TJ-GAL4	Kyoto	104055	GAL4 expression in the somatic support cells	y[*] w[*]; P{w[+mW.hs]=GawB}NP1624 / CyO, P{w[-]=UAS-lacZ.UW14}UW14

Table 2. **Drosophila lines used in feeding assay.** The females mated with during the assay were the wildtype Oregon R. Driver lines used were: GAL4-BAM, GAL4-NOS and GAL4-TJ. The uas line used in crosses was UAS-FOXO.

2.2 QMP application and concentrations

In order to control the level of QMP exposure, QMP concentrations were measured in queen equivalents (QE), with one QE equivalent to the amount of QMP produced by an *A. mellifera* queen in a 24 h period (Beggs et al., 2007 ; Maisonnasse et al., 2010 ; Pankiw et al., 1996). Exposures were established using 13 q.e in all cases, selected as a proven intermediate exposure that allows for detection of an increase or decrease of reproduction in females by adjusting the concentration (Lovegrove., 2019) diluted in 20 µl ethanol, with control exposures comprising the equivalent amount of ethanol.

Each exposure consisted of a diet and either QMP dissolved in ethanol or just ethanol. Diets were created each day in 5mL batches. The liquid diet comprised 95% (v/v) (4.75mL) distilled H_2O, 5% (v/v) (0.25mL) 99.8% absolute ethanol, 0.02 g/mL (0.1g) brewers yeast and 0.03 g/mL (0.15g) sugar. The starvation diet comprised just water and ethanol.

Figure 6. **Modified falcon tube used for experimental exposures.** The base of each tube was removed and replaced with cotton wool. Exposures were pipetted onto two layers of Whatman 1 filter paper in the lid.

QMP was applied to two-layered Whatman 3M filter papers in the lid of modified falcon tubes (Figure 6) as described in Lovegrove et al. (2019). Exposures consisted of 500 µL

of liquid diet or starvation diet and 20 µL of QMP solution or ethanol. For 48h inter-exposure feeding assay (Section 3.3) in all cases 0.5mL of H_2O from each 5mL diet was substituted for 0.5mL of 0.05g/mL aqueous erioglaucine solution. For *Drosophila* activity monitoring (DAM) experiments, the volume of diet was 250 µL due to the use of test tubes rather than falcon tubes. When first trialled with 500 µL, in cases where *D. melanogaster* came into contact with the diet that would be covered and often not survive and so a reduced volume was used.

2.3 Virgin collection

Collection was conducted within set timeframes, based on the ambient temperature of vials, to ensure collected flies were not developed enough to have mated which would impact sexual fitness metrics. This was set at 8 h at 25 degrees and 16 h at 18°C. The collection process consisted of anaesthetising flies on ice and sexing under a dissection microscope based on genital structure and presence of sex combs on the front pair of legs which is only found on males. Following collection, females were kept on media in order to verify that no eggs were laid that produced larva, which would indicate mating prior to collection. No instances of larva production were observed from females post-collection and so no flies are assumed to have mated before collection.

Following collection, male flies were aged for 24 h before exposure in sex-specific vials, with female flies used in aged-matched pairings for mating assays.

2.4 Feeding assays

Post-exposure feeding assays were conducted following a 48 h exposure, to establish the different food consumption levels of male *D. melanogaster* in each exposure type following the exposure period. In feeding assays, an erioglaucine-dyed diet was used to quantify food intake, made by substituting 0.5 mL of distilled water from the liquid diet with 0.5 mL of 0.05 g/mL of an aqueous erioglaucine solution made in 5mL batches, with the resultant diet comprising 85% (v/v) (4.25 mL) distilled H_2O, 5% (v/v) (0.25 mL) 99.8% absolute ethanol, 10% (v/v) (0.5 mL) (0.05 g/mL erioglaucine diluted in H_2O), 0.02 g/mL

(0.1 g) brewers yeast, and 0.03 g/mL (0.15 g) sugar, resulting in a final concentration of 0.005g/mL erioglaucine in the diet.

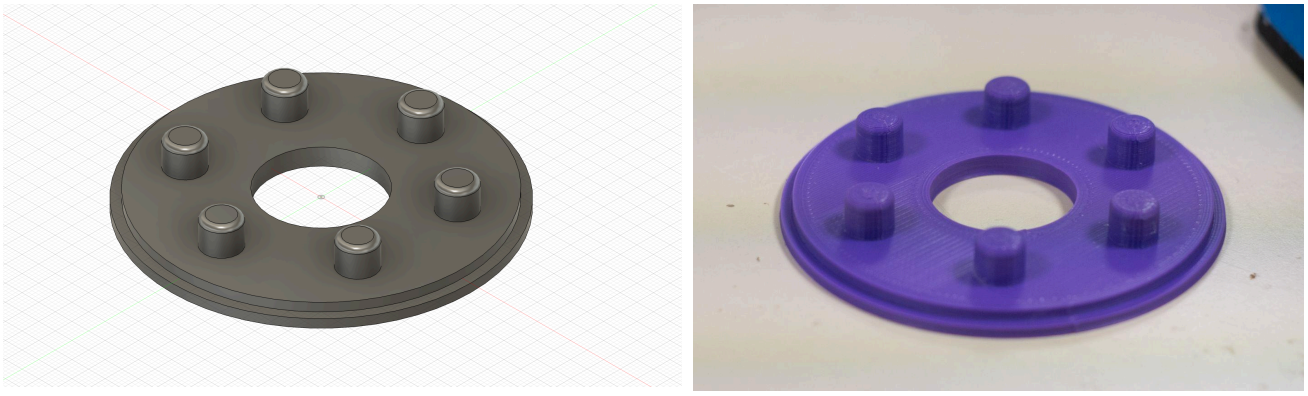


Figure 7. **Rendering and physical view of custom well template designed for feeding assay.** Designed in Fusion360, sliced in Cura and Printed in PLA+ on an Artillery Sidewinder X1

3% agar was poured into petri dishes with custom 3D printed well templates at a 0.8mm depth and 10 mm width, printed in PLA+ (Figure 7). 100µL of dyed liquid diet was pipetted into three of 6 equally distributed wells around the agar. Flies were anaesthetised over ice following exposure to QMP/ethanol/starvation and transferred to the petri dish and allowed to feed for 2 h before being frozen.

In order to quantify the level of food intake, semi-quantitative scoring of the amount of feeding was conducted using visual analysis following freezing, using a scale of 0-3 modified from Lovegrove., (2023) (Figure 9). The scoring system comprised:

0: No dye visible in the abdomen.
1: Dye visible but low in saturation in select places within the abdomen.
2: Dark dye visible in parts of the abdomen however not fully connecting between the dark and light intersection on the back
3: Dark dye found within the majority of the abdomen completely present at the intersection between the dark and light sections of the back.

Scores were assigned to *Drosophila* following the feeding period, with flies anaesthetised on ice, under a dissection microscope with an objective magnification of 1.7x and a 10 x eyepiece magnification.

For a more accurate assessment of food intake, fully quantitative analysis was conducted using optical density analysis. For this, the flies from each exposure group were homogenised in 250µL PBST (1x Phosphate Buffer Saline (PBS), 0.1% Tween-20) and centrifuged. 200µL of each sample was then transferred into a 96-well plate for optical density analysis (Based on the Duncan Lab protocol). Samples were assessed for absorbance across a spectrum (350nm - 750nm) in 10nm intervals, based upon the maximum range of the spectrophotometer used.

2.5 Abdomen colouration analysis

In order to investigate the effect of QMP exposure on abdomen structure, following a 48 h exposure, abdomens were assessed visually using a semi-quantitative scale from 0-2 (Figure 15). The scoring system comprised:

0: Thinly abdomen shaped, no white sections
1: Wider abdomen with white section at the base towards the genitals
2: Wide abdomen with white section towards the top of the abdomen.

Scoring of flies, anaesthetised on ice following exposure, was conducted by comparison to photos taken with a GXCAM imaging camera attached to a microscope set at 1.7x objective magnification with 10x eyepiece magnification. Scoring was conducted blindly, using a custom-developed program in Python that randomly presented images and allowed for scoring whilst keeping the exposure group blind.

2.6 Activity analysis (DAM)

To investigate the effect of QMP on activity, activity was measured over a 24h exposure period from 12am to 12am following an acclimatisation period of ~5h using the TriKinetics infrared-based tri x axial monitoring system, using the DAMsystem3 software. Total

activity was calculated, based on the total activity monitored across the three sets of beams. Activity distribution was analysed by comparison of the relative activity of beams in relation to their position relative to the point of exposure.

Activity was recorded as the total broken beams within five minute intervals, across the assay. Activity from 10 am to 10 pm was considered day activity and 10 pm to 10 am was considered night activity.

2.7 Mating assay

In order to establish the effect of QMP exposure on aspects of sexual fitness, mating assays were conducted, as detailed in Bretman et al., (2010), in test tubes containing modified Bloomington media, with one male and one female per test tube. Age-matched Oregon-R females were transferred before the start of the assay in order to ensure exposed males did not have differing time on the media before exposure to the virgin females. Males were then introduced to the tubes with the time of introduction recorded. Further timings were recorded for the points of mating initiation and mating termination. The duration of the assay was 120 minutes past the time the final male was introduced into the experiment.

Sexual fitness metrics recorded were:
Mating success - Proportion of successful matings in each group.
Mating latency - Time between introduction of the male and female and the start of mating.
Mating duration - the difference in time between the start and end of mating.
Offspring count - Number of offspring produced by each female following mating.
Post-mating fertility - The ratio of offspring production following successful mating.

Following the end of the assay, the males were removed. Females were removed 24 h later. Experimental tubes from mated pairs were kept at 25°C and offspring counts were performed between 12-18 days post-mating to ensure that the F1 generation was fully eclosed without allowing for the F2 generation to eclose.

2.8 UAS-GAL4 manipulation of insulin signalling

In order to assess if low *FOXO* levels in the testes were responsible for the difference in fertility between QMP-exposed male and female *Drosophila,* the GAL4/UAS system was used to look at the mediation of insulin signalling by FOXO expression levels, with drivers selected based on expression in different teste tissue types involved in a range of stages of sperm development. The bag of marbles (BAM) driver line was used to drive FOXO expression in the developing sperm at the late spermatogonia / early spermatocytes stage, a Nanos promoter based (NOS) driver line was used to specify expression in the germ line cells and Traffic Jam (TJ) driver was used to drive expression in the somatic support cells. The range of cell types used allowed for a wider selection of stages of gamete production to be assessed. Crosses were established with greater female numbers than male, with 4~5 females and 3~4 males per cross. Offspring collected for exposure were collected with exposure groups of sizes 12 where possible, specific priority was placed on QMP groups consisting of 12 individuals so as to standardise the dose of QMP where possible between individuals.

2.9 Validation of driver line expression

To verify that driving by the GAL4 lines occurred in the expected tissues, the expression of each line was validated using UAS-mCherry crosses visualised under a confocal microscope, performed by Dr Elizabeth Duncan (Figure 8). Expression was broadly where expected, with germ line cells and somatic support cell expression present predominantly in the apical tip and late spermatogonia / early spermatocytes found further down the testes but before the point of elongation. BAM and NOS expression did see some expression outside of the target tissues, with both showing mCherry expression at lower levels in elongated spermatids.

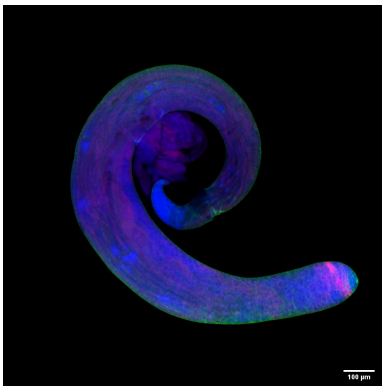
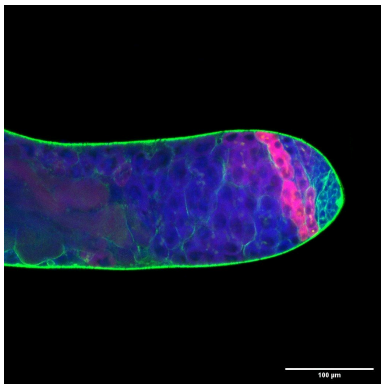
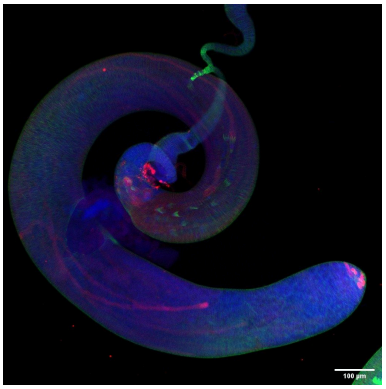
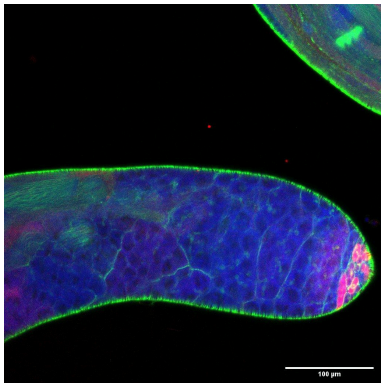
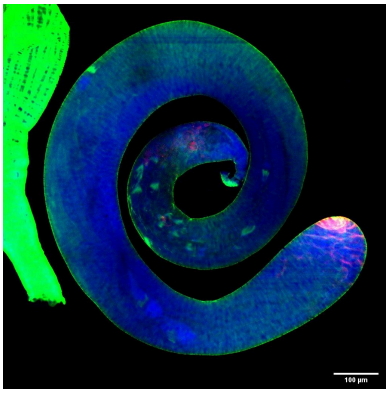
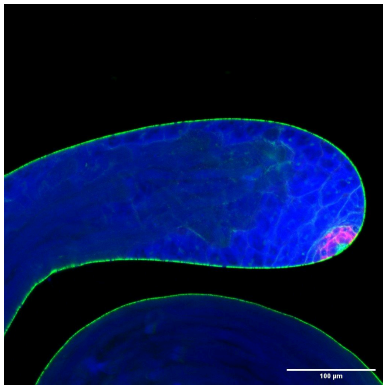
Line (Identifier)	Location of expression	Expression across testes	Expression at apical tip
BAM	Late spermatogonia / early spermatocytes		
NOS	Germ line		
TJ	Somatic support cells		

Figure 8. **GAL4 drivers used in mating assay.** Crosses and visualisation performed by Dr Elizabeth Duncan. All images are a single section. Cells were stained with DAPI (blue, stains nuclei) , Phalloidin-48 (green, stains actin) and mCherry (red, expressed in response to GAL4 driving thus indicative of areas of expression, indicative of the areas that FOXO will be expressed in in the FOXO crossed lines used in the mating assay).

2.10 Data handling and Statistical analysis (R)

Assay-generated data was stored in Google Sheets before being imported into RStudio (R Core Team, 2024). Data was processed and formatted using the “Tidyverse” V2.0.0 package for data frame generation (Wickham et al., 2019), data processing and pipe operations; “Janitor” V2.2.0 for cleaning of variable names (Firke, 2021); and custom functions to filter rows.

All graphs were generated in RStudio using the “ggplot2” V3.5.1 package (Wickham, 2016). The complimentary package “patchwork” V1.3.0 (Pedersen, 2024) was used for generating multi graph plots, with “ggsignif” V0.6.4 (Ahlmann-Eltze and Patil, 2021) or “geom_text” (from ggplot2) used for specifying significance. Further details on these packages can be found via the CRAN database.

Statistical tests were conducted using cumulative link models (CLM) compared to null models for ordinal data using the “ordinal” package (Christensen, 2022), with significance assigned in cases where factors provided significantly better explanation of the distribution than the grouping of the null model. For non-ordinal categorical data, chi-squared tests or Fisher’s exact tests were conducted based on sample size, with Fisher’s exact test used for low sample size data. For scalar data, anova analysis was conducted with pairwise post hoc testing using TukeyHSD.

Section 3 - Results

3.1 Feeding level does not differ, regardless of the method of testing, when measured post-exposure in male QMP-exposed Drosophila.

In order to determine if QMP exposure impacts feeding in *D. melanogaster* males, as is seen in *D. melanogaster* females (Lovegrove et al., 2023), post exposure feeding assays were performed, consisting of an initial 48h exposure to one of three diets (liquid diet with ethanol, liquid diet with QMP or a starvation diet with ethanol) and a subsequent exposure to a dyed liquid diet for 2h. Following this, flies were scored both semi-quantitatively using a scale and fully-quantitatively using an optical density spectrophotometer to quantify the level of food intake during this period.

3.1.1 QMP does not affect feeding when assessed post exposure (using a semi-quantitative scale)

Following the method of Lovegrove et al. (2023), who showed that females exposed to QMP subsequently eat more, the same methodology was used with a slightly modified scoring system (Figure 9), to investigate whether males are similarly affected, with 48 h exposures to QMP-Diet, diet or liquid controls prior to a 2 h feeding window.

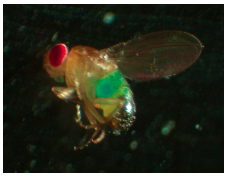
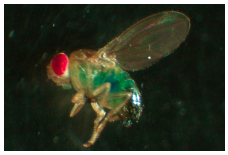
Score/ Orientation	0	1	2	3
Side				
Upright				

Figure 9. **Semi-quantitative feeding scale**: Flies are scored in a range of 0-1 based on dye distribution within the abdomen. 0: No dye visible in the abdomen, 1: Dye visible but low in saturation in select places within the abdomen, 2: Dark dye visible in parts of the abdomen however not fully connecting between the dark and light intersection on the back, 3: Dark dye found within the majority of the abdomen completely present at the intersection between the dark and light sections of the back. Images visualised using an attached GXCAM imaging camera with 1.7x objective and 10x eyepiece magnification.

In combination diet and exposure type were found to be significant predictors of semi-quantitative feeding score (AIC = 376.82) when modelled against a null model using clm (AIC = 507.33, X^2 = 136.52, df = 3, p = 2.2e-16). Meanwhile, as an individual factor, exposure type (QMP / ethanol exposure) was not found to be a significant factor in the mediation of the semi-quantitative value distribution (AIC = 509.33), not being found to differ significantly when using a clm test from a null model (AIC = 507.33, X^2 = 0.002, df = 1, p = 0.9642). Diet (AIC = 376.20) was found to have significant impact on the distribution when compared to a null model using clm analysis (AIC = 507.33, X^2 = 133.13, df = 1, p = 2.2e-16). These results suggest that the presence or absence of food in the diet is significant but that the exposure to QMP is not (Figure 10), which is in contrast to the findings in females, which found statistical differences between QMP-exposed and Ethanol-exposed *Drosophila* (Lovegrove et al., 2023).

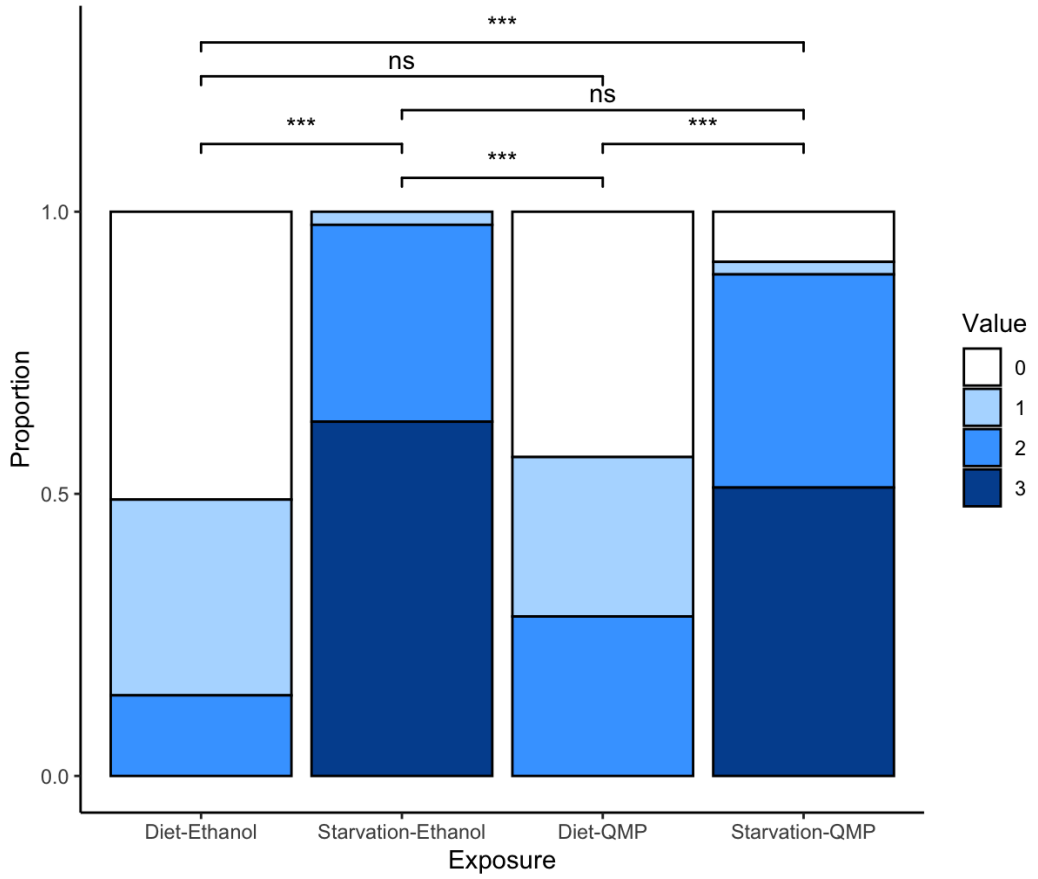


Figure 10. **QMP does not impact feeding post eclosure in *Drosophila*.** Semi quantitative comparison of dye distribution in the abdomen following 48 h exposures of diet, starvation and QMP exposed *Drosophila*. Ranking system modified from (Jiang et al., 2018). Scoring values as described in Figure 9. Statistical analysis was conducted using clm comparisons with significance being determined at $p < 0.05$ relative to a null model. Significance notations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
N = 183 (Ethanol Diet = 49, QMP Diet = 46, Ethanol Starvation = 43, QMP starvation = 45).

3.1.2 Establishing a fully-quantitative feeding quantification assay

Following semi-quantitative analysis, fully quantitative analysis was conducted.This allowed greater resolution, removal of subjective result generation and the ability to differentiate between differing levels of intake at the higher consumption levels.

3.1.3 Validating the concentration of eryoglycine to result in a linear range of light absorption

The ability to differentiate between the consumption levels relies on the absorption values lying within a linear range so that an increase in dye level results in a linear increase in absorption. Previously conducted optical density analysis using eryoglycine in *Drosophila* used a wavelength of 630 nm for absorbance analysis (Jiang et al., 2018). In order to validate this wavelength and determine concentration of dye in the sample for appropriate optical density analysis, a serial dilution was performed and absorbance measured across these ranges (Figure 11).

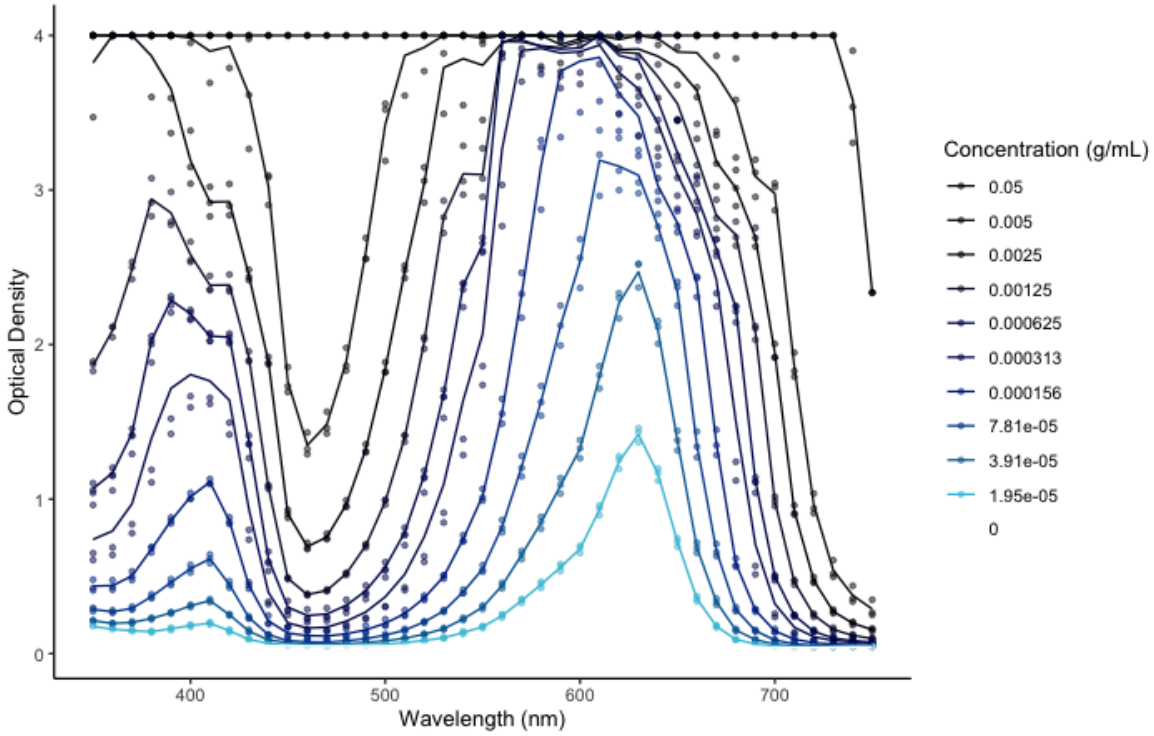


Figure 11. **Selection of wavelength and concentration for eryoglycine in liquid diet.** Based on optical density analysis across a spectrum for samples in a serial dilution, measured using a 96-well plate spectrophotometer. Concentration measured in g/mL. Line plotted along the mean value of 3 replicates, with points representing individual data points.

An absorption value of 630 nm was confirmed, based on the verification of an optical density peak at this wavelength (Figure 11). The concentration was trialled at 0.005 g/ml in the dyed food and further diluted in the optical density sample preparation stage.

3.1.4 Spectrum Visualisation (Conformation of point absorbance value)

In order to validate the concentration and wavelength in samples, large spectrum range analysis was conducted on samples from different exposures in order to assess the deviation between samples. The deviation around the point of 630 nm and the absorbance falling within the linear range validated the use of this wavelength and concentration for the point analysis (Figure 12).

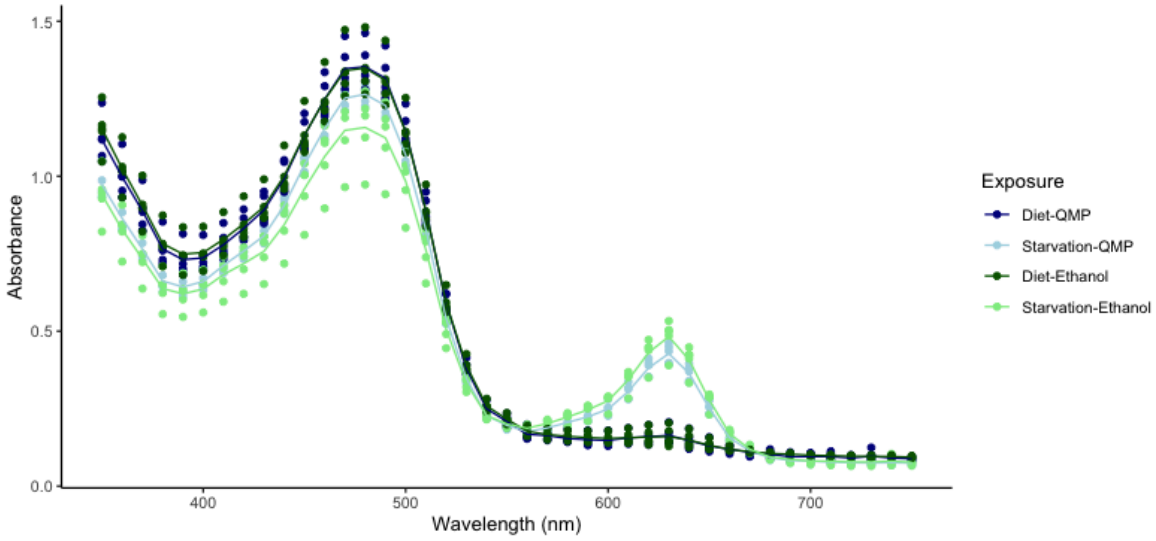


Figure 12. **Full spectrum graph of Diet, Starvation and QMP exposed *Drosophila*.** Ethanol exposed data points are marked in green, QMP exposed data points are marked in blue. Data points from *Drosophila* fed a liquid diet are high in saturation, data points from starvation exposure are low saturation, measured using a 96-well plate spectrophotometer. Line plotted along the mean value of 5 replicates, with points representing individual data points.

3.1.5 Post-exposure feeding levels of QMP exposed male *D. melanogaster* are determined by diet not QMP exposure in male *D. melanogaster* (Point absorbance at 630 nm)

In order to investigate the absolute differences between exposure groups with regards to post exposure feeding, the point absorbance was measured using an optical density spectrophotometer to give a relative value of erioglaucine which serves as a proxy for feeding level.

The combination of exposure and diet were found to have significant impact on the post-exposure food consumption levels when used as predictive factors compared using a null model ANOVA (F(3, 16) = 104.1, p = 1.042e-10) (Figure 13). However, as in the semi-quantitative analysis, the major influence was diet, with TukeyHSD post hoc testing finding significant difference between individual exposures in all cases when the diet component within them differed, regardless of QMP exposure (See Appendix 6.1). In contrast to what has been found in female *Drosophila* (Lovegrove et al., 2023), no significant difference was found without difference in diet between groups. However, Lovegrove did not analyse using fully quantitative optical density analysis.

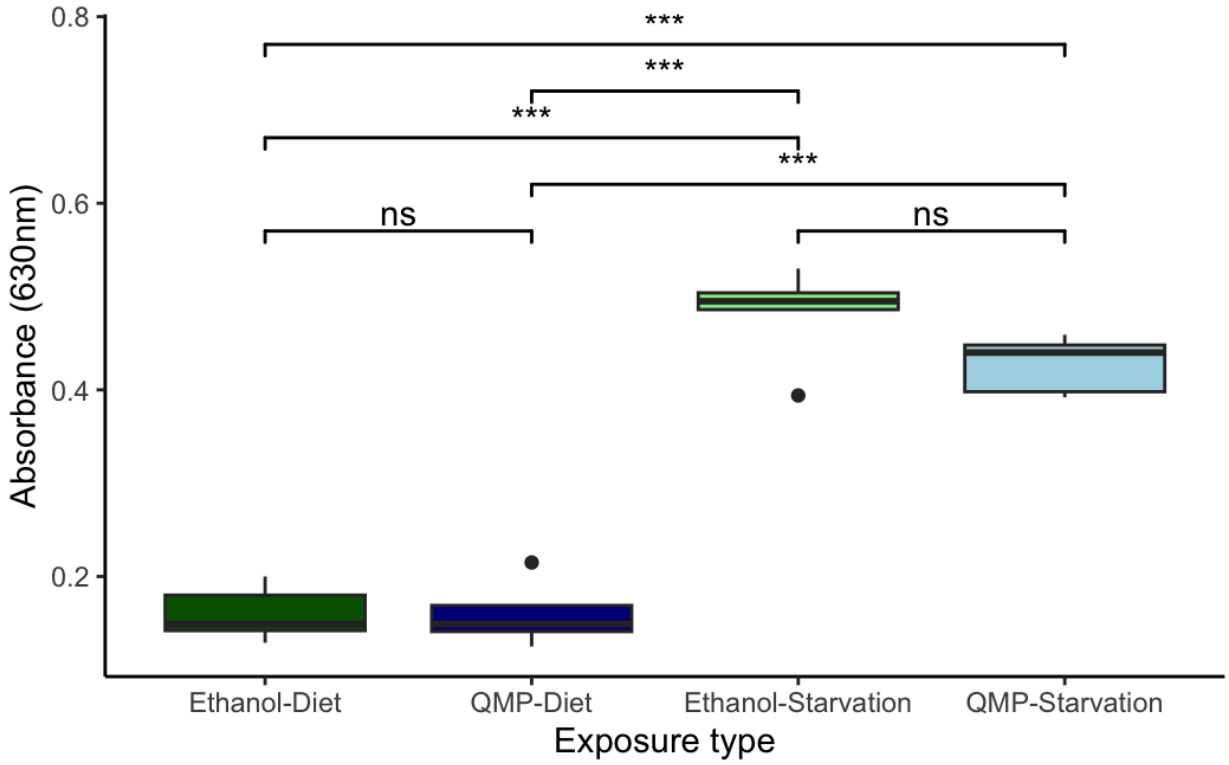


Figure 13. **QMP does not effect feeding levels post exposure.** Food intake of *Drosophila* following a 48h exposure to either 13 Qe QMP on a liquid diet or ethanol with either a liquid diet or a starvation diet. Absorbance across different exposure groups at 630nm wavelength was measured using a 96-well plate spectrophotometer, indicating eryoglycine intake, which acts as in indicator of relative food intake. Statistical analysis was conducted using ANOVA comparisons. Post-hoc pairwise comparisons were performed using TukeyHSD analysis. Significance notations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N = 5 Replicates.

3.2 QMP-exposed male Drosophila have a distinct abdomen colouration

During post-exposure feeding analysis, it was observed that QMP exposured *Drosophila* males often had abdomen with a “milky” colouration (Figure 14B). In order to assess the cause, abdomen dissections were conducted and found cloudy crops in these individuals (Figure 14C).

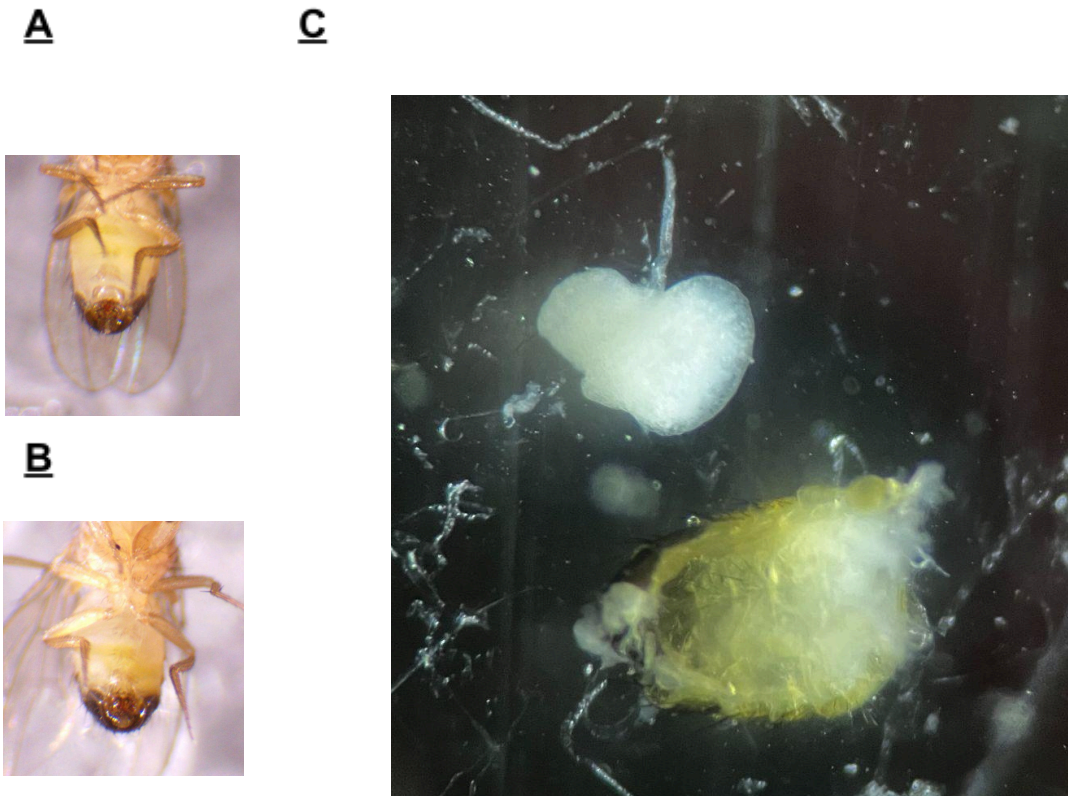


Figure 14. **Milky colouration of QMP exposed abdomen and crop.** (A) Image of non QMP exposed abdomen,(B) QMP exposed abdomen, (C) QMP exposed crop in male *Drosophila* top of image abdomen bottom of image. Images visualised using an attached GXCAM imaging camera with 1.7x objective and 10x eyepiece magnification.

Following these observations, semi-quantitative analysis was conducted on QMP, starvation and diet-exposed *Drosophila* in order to quantify the phenotypic differences between groups, scored blindly using a custom-written python program.

QMP exposure was found to significantly impact the visible colouration of the abdomen of male *Drosophila*, with a greater number of individuals with abdomens scored at a value of 2 (Figure 15). Fisher's exact tests found a difference in the distribution across between groups of (p = 6.731e-10). Post hoc testing found significant differences between QMP and both Diet (p = 1.034e-06) and Starvation (p = 9.663e-10) but not between diet and starvation (p = 0.3405), indicating that the difference is due to QMP exposure rather than differing diets.

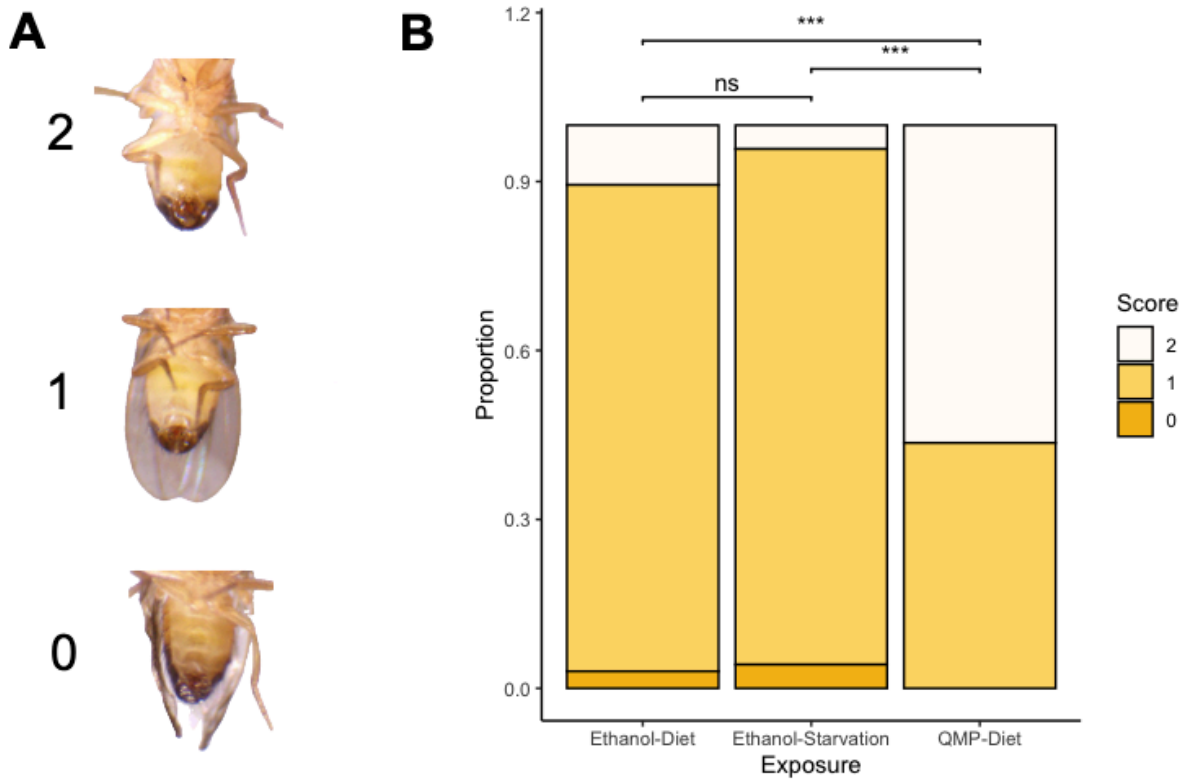


Figure 15. **QMP exposure changes the visible colour profile of the abdomen.** Stacked box plot of scoring across groups of exposed *Drosophila*. Scored on a semi quantitative scale as specified in section 2.5. Statistical analysis was conducted using a Fisher's exact test. Significance notations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Images visualised using an attached GXCAM imaging camera with 1.7x objective and 10x eyepiece magnification. N = 176 (Ethanol Diet = 66, Ethanol Starvation = 71, QMP Diet = 39), six replicates.

3.3 QMP exposed male *Drosophila* eat more during the duration of exposure.

The lack of difference between diet and QMP exposed male *D. melanogaster,* when measured post exposure, combined with the different crop phenotypes found frequently between these groups, gave plausibility to the idea that feeding may be different during the exposure period rather than following it (as tested in section 3.1.1 and 3.1.5). In order to test this, the exposure diets were substituted for dyed diets (outlined in section 2.2) and the same feeding consumption metrics were recorded following the 48h exposure period.

Semi-quantitative analysis of intra-exposure feeding found no significant impact of QMP on feeding, with no significance found when comparing between groups (AIC = 101.954) using a clm against a null model (AIC = 99.163, $X^2 = 1.2087$, df = 2, p = 0.5464) (Figure 16). This is in contrast to the finding of the post-exposure semi-quantitative feeding analysis but is likely due to the much longer time available for feeding, resulting in a large proportion of abdomens scoring high across all exposures, meaning that fully quantitative analysis is a more suitable measurement in this instance.

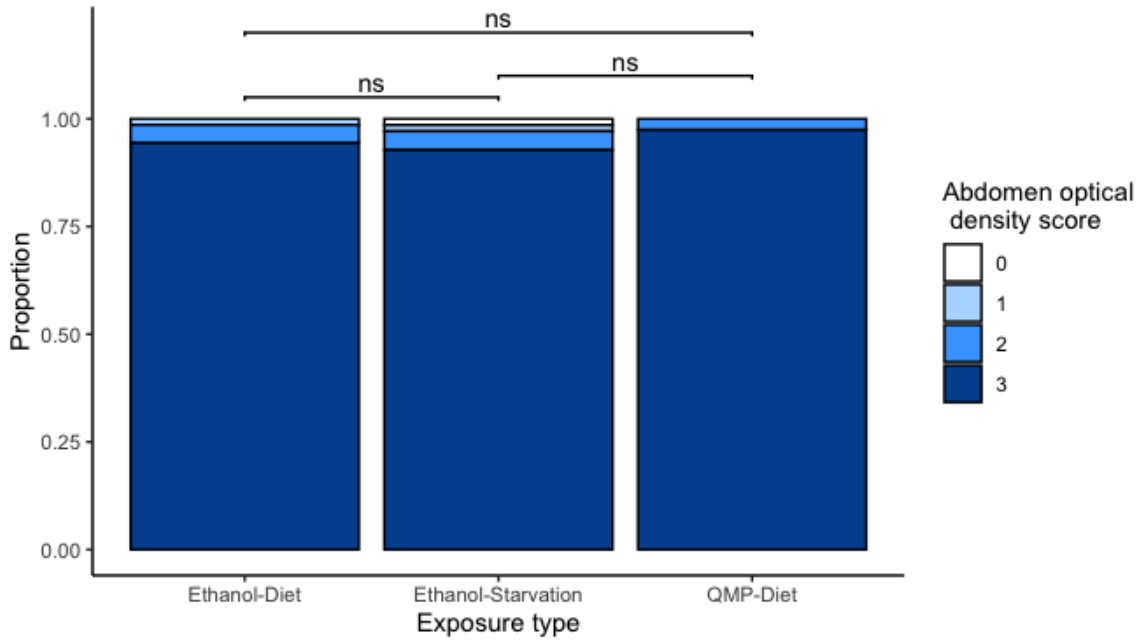


Figure 16. **QMP exposure does not alter the intra exposure food consumption of *Drosophila* when assessed using a semi quantitative scale.** Stacked bar chart of semi quantitative analysis of abdomen dye colouration in *Drosophila* following 48h liquid diet, QMP and starvation exposures all consisting of 0.05% erioglaucine. Statistical analysis was conducted using clm analysis with significance determined at $p < 0.05$ when compared to a null model. Significance notations: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. N = 204 (Ethanol Diet = 72, Ethanol Starvation = 72, QMP Diet = 60, replicates = 6.

For the fully quantified comparison, the point absorbance was compared between treatments at 630 nm, as validated by the wide spectrum absorbance analysis conducted on the erioglaucine dye solutions, in order to quantify the level of diet ingested during the course of the exposure period in each exposure type.

Feeding levels during exposure were found to be significantly affected by exposure type, assessed by ANOVA (F(2, 12), p = 6.254e-05) (Figure 17 (Left)). QMP exposure was found to result in significantly higher levels of dye intake when assessed using TukeyHSD pairwise comparison than Diet (p = 2.138534e-04, C.I. = 0.09288222, 0.24859778) and Starvation (p = 1.323019e-04, C.I. = -0.25780222, -0.10208667) exposure. However, no difference was found between intake in Diet and Starvation exposures (p = 0.9468811653). These results show that QMP exposure increases food intake during the exposure period. This contrasts the findings of the post-exposure semi-quantitative analysis in which the only factor that affected feeding levels was diet and not QMP exposure (Section 3.3.1). These results of QMP exposure are consistent with the findings of Lovegrove et al. (2023). However, Lovegrove's results were collected post-exposure rather than intra-exposure.

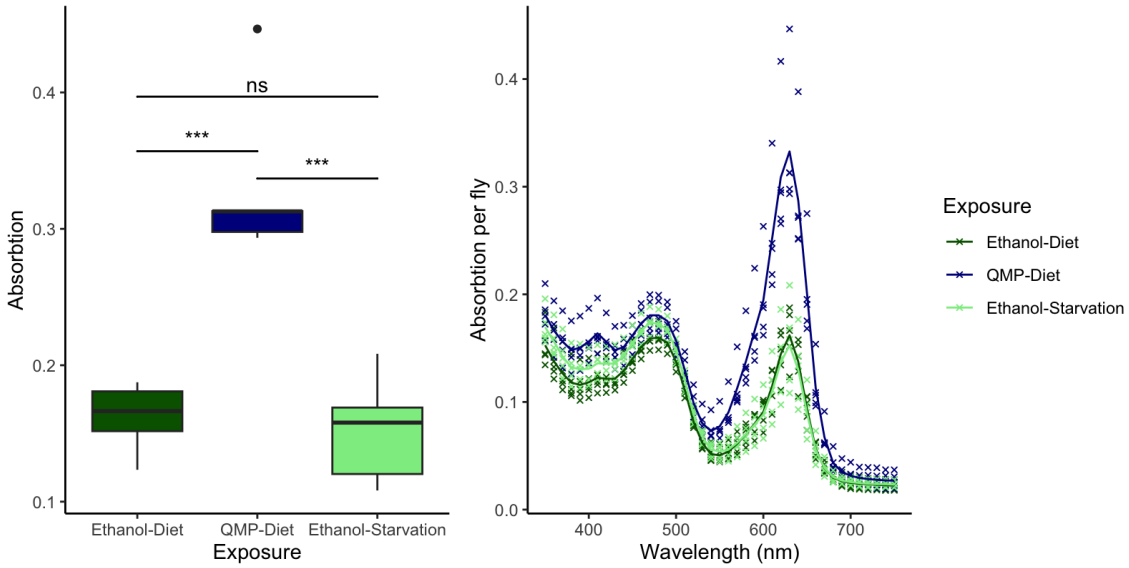


Figure 17 (Left). **QMP increases intra exposure feeding.** Optical density analysis at 630nm, quantifying the feeding activity of Diet, Starvation or QMP exposed *Drosophila* during the exposure period. Statistical analysis was conducted using ANOVA comparisons. Significance notations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
(Right) **Full spectrum optical density analysis**, quantifying the intra exposure feeding activity of Diet, Starvation or QMP exposed *Drosophila* over a 48 h exposure period. N = 5 replicates.

3.4 QMP causes excess mortality in male *D. melanogaster.*

In order to test the effect of QMP on mortality the survival rate of exposed *D. melanogaster* males was recorded following a 48h exposure to one of three diets.

Mortality was significantly impacted by QMP exposure when analysed using Fisher's exact test (p = 4.535e-12) (Figure 18). Mortality was found to increase in QMP-exposed *Drosophila* when compared to starvation and diet-exposed groups. Pairwise Fisher's exact tests showed significant differences between Diet and QMP (p = 7.648e-09) and Starvation and QMP (p = 1.319e-07) but not between Diet and Starvation (p = 0.4965).

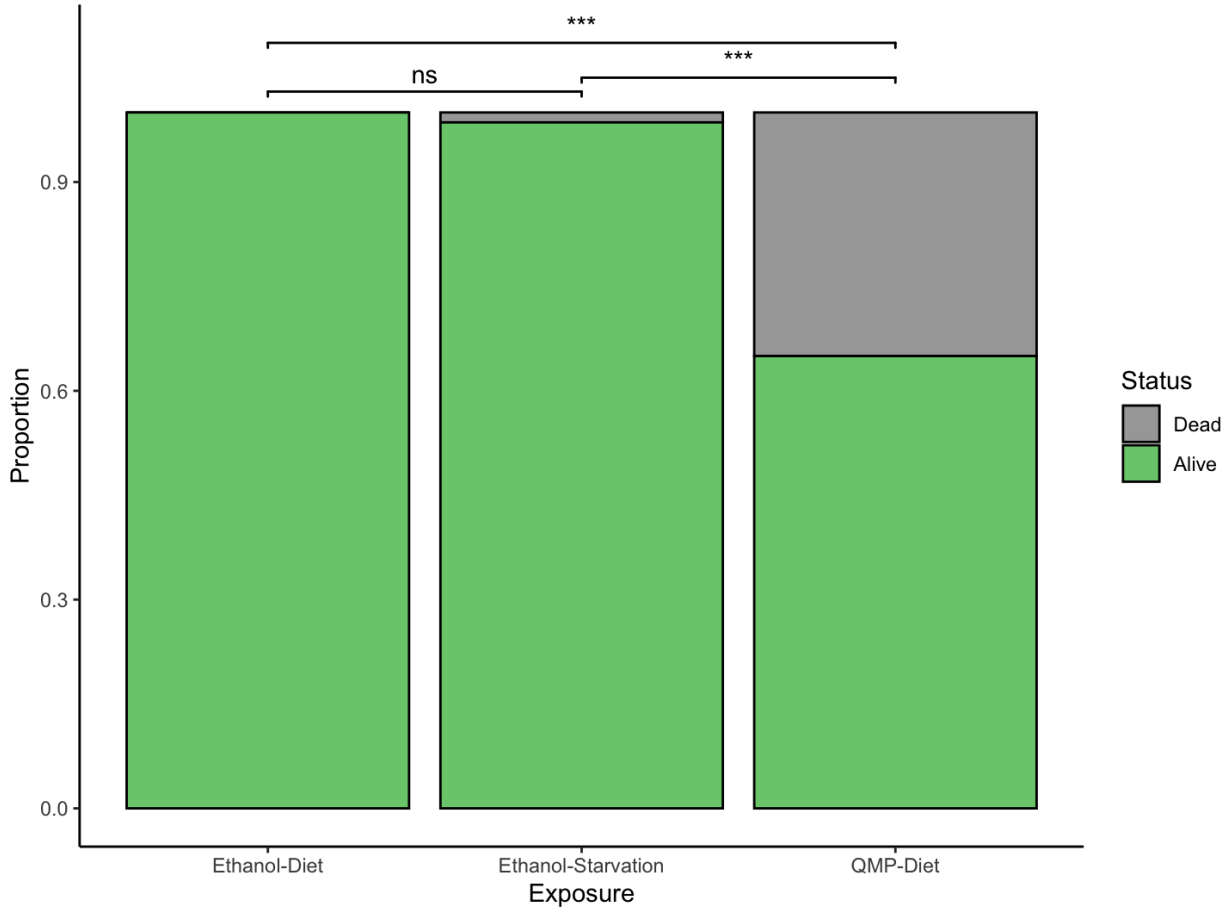


Figure 18. **QMP exposure results in a higher death rate.** Stacked box plot displaying the survival ratios across treatment groups: Diet, QMP and Starvation exposures. Statistical analysis was conducted using a Fisher's exact test. Significance notations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N = 204 (Ethanol Diet = 72, Ethanol Starvation = 72, QMP Diet = 60), replicates = 6.

3.5 QMP alters activity level and distribution in male *Drosophila*

QMP is known to affect locomotion in honey bees (Beggs et al., 2007). In worker bees, a retinue response is induced, and one component of QMP (9-ODA) is known to attract drones to localise towards it. Male *Drosophila* have been shown to orient towards QMP in a T-maze (Croft et al., 2017). However, no studies have looked at the effect of QMP exposure on absolute mobility. In this experiment mobility was assessed during the duration of QMP exposure, with scoring based on the number of times infrared beams were broken as *Drosophila* passed through the test tube in 5 minute intervals.

3.5.1 QMP exposure was found to reduce activity compared to all non QMP-exposed groups across day and night.

To test the effect of QMP exposure on activity three exposures were prepared (as outlined in section 2.2). 10 *D. melanogaster* males were placed in each exposure and their activity was recorded over 24h using the TriKinetics infrared-based tri x axial monitoring system.

Exposure type was found to have a significant impact on the total activity of male *Drosophila* when compared against a null model using an ANOVA ($F(2, 3924) = 350.1$, $p = 2.2e-16$) (Figure 19). QMP-exposed flies had significantly lower activity than Ethanol-Diet and Ethanol-Starvation diets in both the day (Diet: p = 2.072291e-08, C.I. = -42.584705, -26.066321, Starvation: p = 2.072291e-08, C.I. = -50.457139, -33.938755) and the night (Diet: p = 2.072291e-08, C.I. = -96.371399, -79.143752, Starvation: p = 2.072291e-08, C.I. = -55.736631, 38.508983), compared using pairwise Tukey analysis. These results indicate that QMP reduces activity in both the day and the night compared to both fed and starved non QMP-exposed groups.

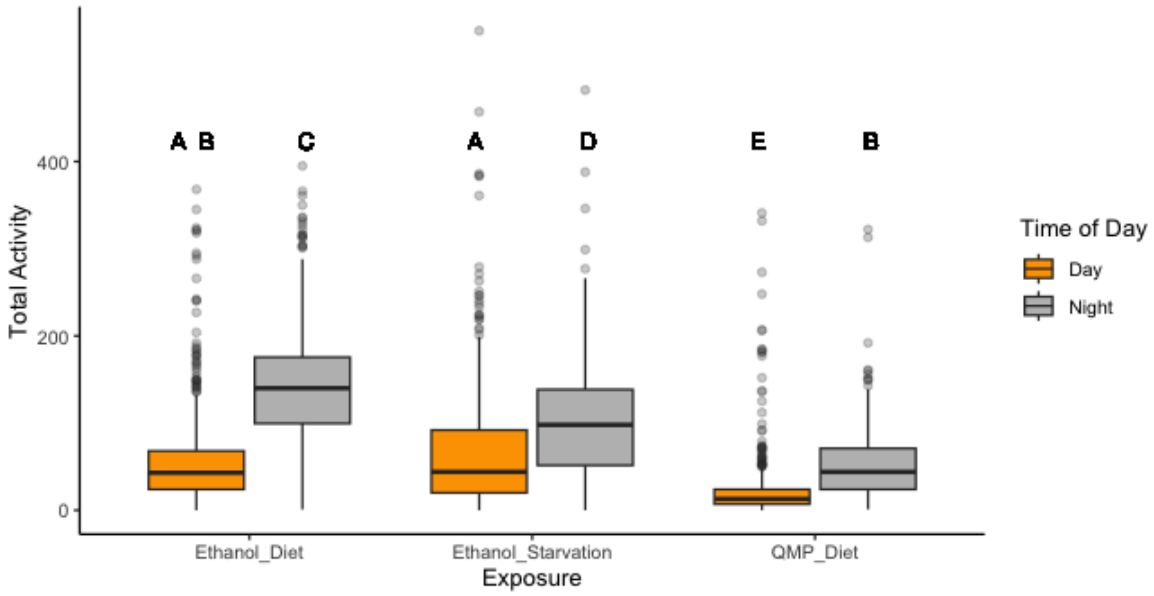


Figure 19. **QMP exposure reduces activity in male *Drosophila*.** Total activity was recorded by the total amount of readings across three laser beam rings along the tube in five minute lots over 24h of exposure. Data collected across five replicates using TriKinetics - Drosophila Activity Monitoring System. Boxes not sharing a letter are significantly different from one another at a significance of $p < 0.05$. N = 5 replicates. 10am - 10pm categorised as day and 10pm-10am categorised as night.

3.5.2 QMP exposure affects distribution of *D. melanogaster* males

Activity was measured across three beams that circle the circumference and were distributed along the length of the tube. This facilitated analysis of the activity variance at points at varying distances away from the exposure point. These results were not considered as a proxy for feeding due to the inability to distinguish where *Drosophila* were between recorded points of activity.

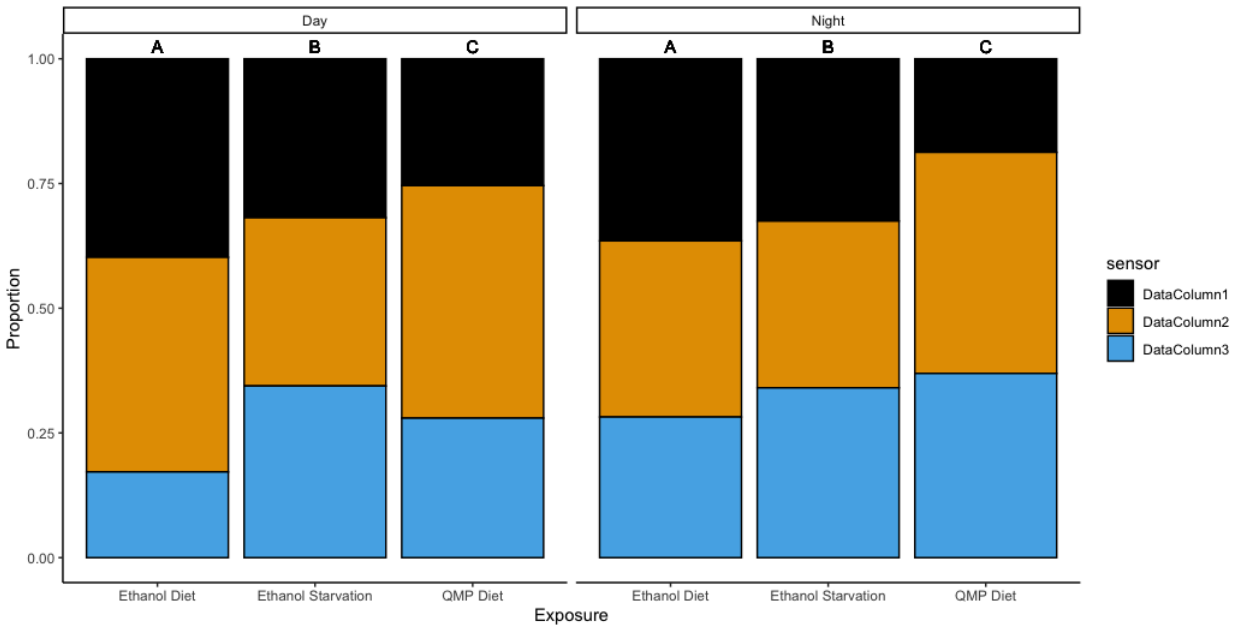


Figure 20. **Activity distribution shown for each replicate and exposure combination in both day and night conditions.** Activity distribution was calculated through the use of activity values for thee sensors sets down the length of the test tube expressed relative to the total activity of the time frame. Data recorded over 24h of exposure. Data collected from five replicates. Boxes not sharing a letter are significantly different from one another at a significance of $p < 0.05$. The frequency of activity at each of three rings spaced along the tube around the circumference are represented by different colours. N = 10 *D. melanogaster* per tube per exposure, 5 replicates.

Total values for activity across each sensor beam were combined in order to give total activity values for each exposure in each area. Exposure was found to have a significant impact on activity across the day ($X^2(4, N = 9) = 3692.5$, $p = 2.2e\text{-}16$) and night ($X^2(4, N = 9) = 3792.5$, $p = 2.2e\text{-}16$) when compared using a chi squared test (Figure 20).

QMP exposure was found to significantly affect the distribution of activity within the enclosure compared to both Diet-Ethanol and Diet-Starvation exposures, with post hoc

testing with pairwise chi squared tests finding significant differences between all pairs of exposures in day and also night results ($p = 2.2e-16$).

3.6 Mating assay - The effect of QMP on tissue-specific insulin signalling

The fertility of male *Drosophila* has been found to be insensitive to QMP (Chambers., 2023) despite QMP reproductively repressing females (Camiletti et al., 2016 ; Lovegrove et al., 2019). In female *Drosophila*, QMP exposure induces a starvation-like effect, depressing insulin signalling in the ovaries (Lovegrove et al., 2023). It is currently unknown if the dichotomy found between males and females is due to differential insulin signalling between males and females or due to different genital tissue responses to the induced signalling (Chambers, 2023).

The genitals of male *Drosophila* are known to be insulin insensitive. Prior study has shown that low levels of *FOXO* in the tissue render them insulin insensitive (Tang et al., 2011) and that increasing *FOXO* levels in the genitals restored nutritional plasticity in this tissue and subsequently reduced multiple sexual fitness metrics. If the dichotomy is a result of male-specific genital insulin signalling insensitivity, as these observations suggest due to FOXO being part of the insulin signalling system, then elevation of *FOXO* levels in the genitals may result in QMP dependent fertility.

In order to assess the level of mediation of the effect of QMP by *FOXO*, individuals with specific *FOXO* expression were produced by mating male GAL4 lines with virgin female UAS FOXO lines, resulting in tissue-specific FOXO production. Three drivers were selected: BAM, NOS and TJ which drive expression in three different tissue types in the male genital tissues (late spermatogonia / early spermatocytes, germ line cells and somatic support cells respectively).

Following driver selection, expression locations for each driver were subsequently visualised (Figure 8) using confocal microscopy with driver x MCherry crosses, performed by Dr Elizabeth Duncan.

In order to manipulate FOXO expression, crossed flies with a paternally-inherited driver line and a maternally inherited UAS-FOXO line, were exposed to QMP before being assessed in a mating assay. This allowed for identification of the effect of male cell

type-specific *FOXO* expression in the testes on sexual fitness after QMP exposure. Multiple aspects of sexual fitness were assessed from the mating assay. Including: Mating success, Mating latency, Mating duration, Offspring count and Chance of offspring production.

3.6.1 Proportion mated

3.6.1.1 QMP does not affect proportion mated in *D. melanogaster* males with FOXO expression in Late spermatogonia / early spermatocyte cells (BAM-GAL4 driven)

Overexpression of FOXO in the *D. melanogaster* testis using the BAM promoter showed a significant reduced in the proportion of pairs mating, with BAMxFOXO found to significantly differ from the parental control line (BAMxBAM) in QMP-Diet ($p = 8.661e-07$), Ethanol-Diet ($p = 5.446e-10$) and Ethanol-Starvation ($p = 4.272e-06$) when compared using Fisher's exact testing, reducing the percentage mating from 63% in the parental control compared to 2% in the BAMxFOXO cross (Figure 21).

However, there was no significant difference found in mating proportion between exposure types within each cross when compared using Fisher's exact test in either cross, indicating that FOXO expression in the late spermatogonia and early spermatocytes does not affect mating proportions in these conditions. However, so few male *Drosophila* mated in the BAMxFOXO cross that differentiation between the rates in each exposure would not be possible, even if a difference in levels of fertility is induced between treatments.

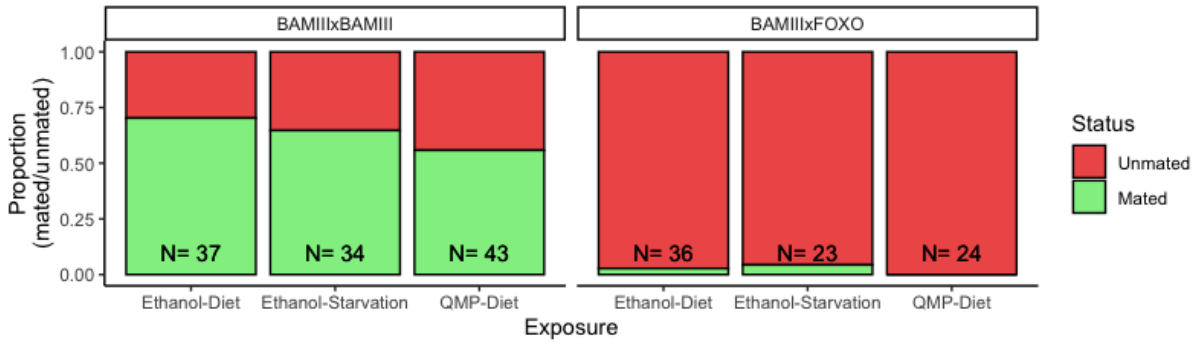


Figure 21. **BAMxBAM and BAMxFOXO proportion mated.** Proportion of male *Drosophila* which mated within a 120 minute mating assay exposed to a single Oregon R female, following a 48h exposure to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

3.6.1.2 QMP does not affect mating proportion in *D. melanogaster* males with Germ line cell expression of FOXO (NOS-GAL4 driven)

In contrast, overexpression of FOXO in the germ line cells under the control of the NOS promoter was found to significantly increase the proportion of mating relative to the NOSxNOS parental control cross across all exposures when compared using a Fisher's exact test, increasing the average proportion mating across exposures from 26% to 78%. This was consistent across all exposure types (QMP-Diet ($p = 0.001746$), Ethanol-Diet ($p = 3.105e-06$) and Ethanol-Starvation ($p = 0.04091$)) (Figure 22).

Exposure type was not found to result in significant difference between groups within crosses in either cross. This indicates that the expression of FOXO in the germ line cells does not affect the impact of QMP exposure on mating proportion.

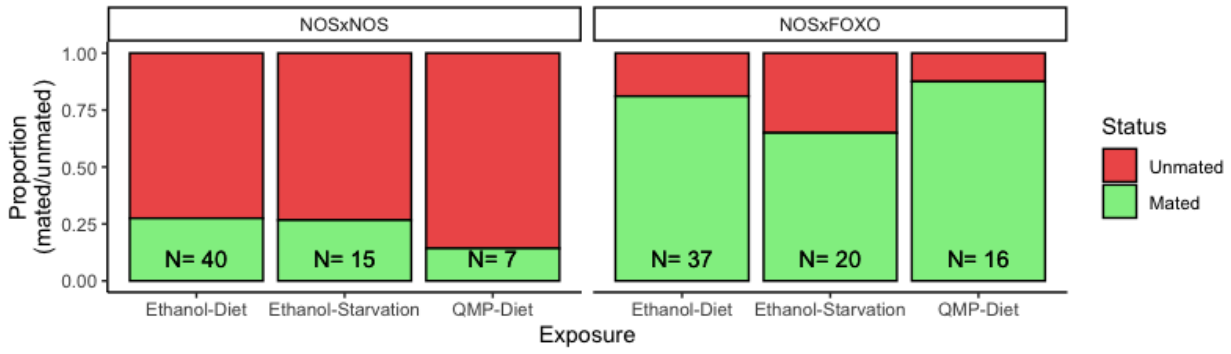


Figure 22. **NOSxNOS and NOSxFOXO proportion mated.** Proportion of male *Drosophila* which mated within a 120 minute mating assay exposed to a single Oregon R female, following a 48h exposure to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

3.6.1.3 The effect of QMP on mating proportion in *D. melanogaster* males is dependant on the expression of FOXO in the Somatic support cell (TJ-GAL4 driven)

The expression of FOXO in the somatic support cells specified by the TJ promoter was found to significantly reduce mating proportion relative to the parental control line TJxTJ in both Ethanol-Diet (p = 0.001913) and Ethanol-Starvation (p = 0.0005449) but not in QMP-Diet exposure (p = 0.2376) (Figure 23).

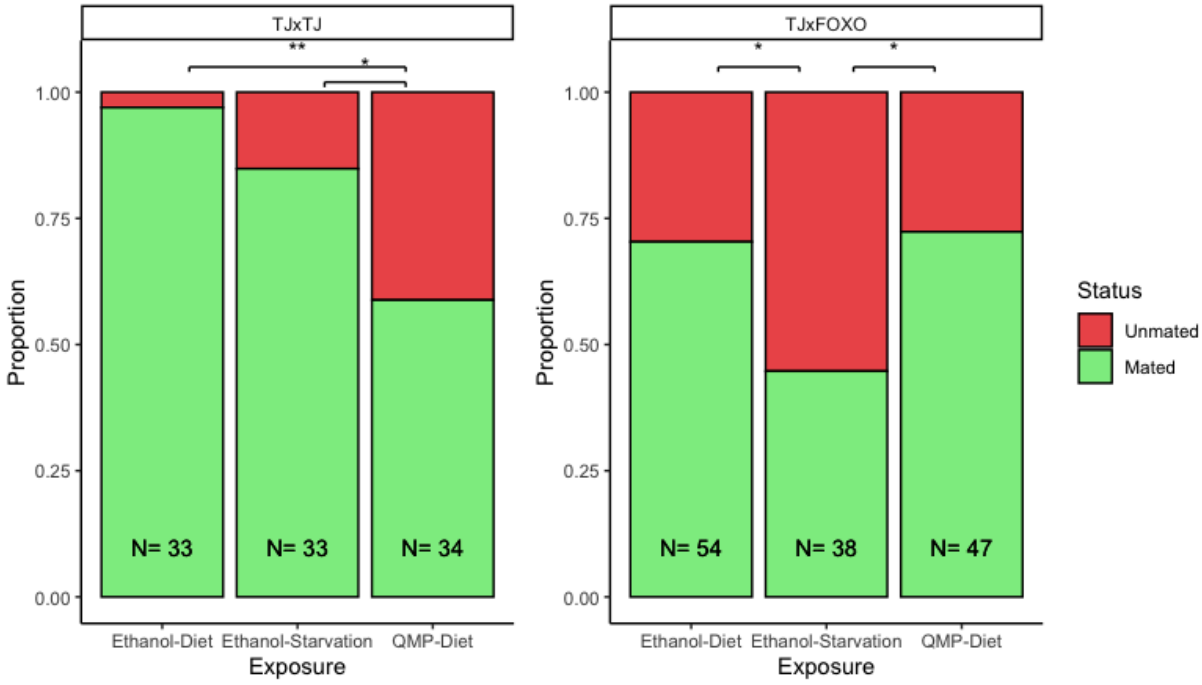


Figure 23. **Pairwise comparisons of effect of exposure on mating proportion in TJxTJ (left) and TJxFOXO (right).** Proportion of male *Drosophila* which mated within a 120 minute mating assay exposed to a single Oregon R female, following a 48h exposure to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

Rates of mating were found to be significantly affected by exposure type in both the FOXO expressing TJxFOXO cross(p = 0.01598), and in the non FOXO expressing parental control line TJxTJ (p = 0.0003697) when compared using Fisher's exact testing. In the TJxFOXO cross, QMP-Diet exposure was found to differ significantly from Ethanol-Starvation (p = 0.01787) exposure but not from Ethanol-Diet exposure (p = 0.9089549). Conversely, the TJxTJ line, which does not express FOXO, found significant difference in mating rates between QMP-Diet and both Ethanol-Diet (p = 0.0002217) and Ethanol-Starvation (p = 0.02912) when compared with pairwise Fisher's exact analysis.

These results indicate that the TJ parental control line is sensitive to QMP exposure despite not having GAL4 driven FOXO expression, but that driving FOXO expression in the somatic support cells changes the nature of the response to QMP. Whilst the diet and starvation exposures experience reduced mating rates following expression of FOXO in the somatic support cells, the QMP-exposed groups do not. This reduced impact of FOXO expression on the decrease of mating rates in QMP-exposed individuals relative to starvation-exposed and diet control groups indicates that the effect of QMP exposure on mating seems to act independently of the level of FOXO expression in these cells, but also that further reduction in ability to mate is not induced by expression of FOXO. Is it possible that in flies of this genetic background the response to QMP is mediated by increased FOXO signalling in the somatic support cells which reaches a threshold such that further increasing the FOXO expression results in no change in mating.

3.6.1.4 QMP exposure has no impact on mating rates in the FOXO control line

In the UAS-FOXO parental control line, which has no GAL4 expression and therefore should be expected not to express FOXO in a driven way, no significant impact was found from QMP exposure or starvation relative to the control diet group (Figure 24). These results are in line with the findings of the wildtype line OregonR (Chambers, 2023), indicating that in this genetic background with no known overexpression of FOXO in any testes tissue, QMP exposure does not result in altered successful mating.

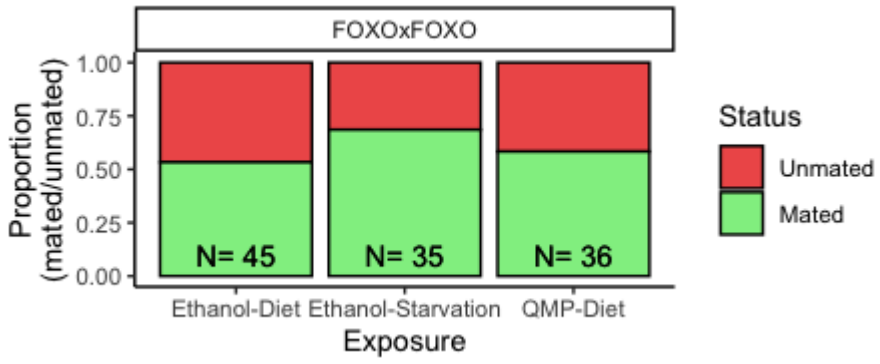


Figure 24. **Mating proportion is not significantly impacted by QMP exposure in FOXOxFOXO male *Drosophila*.** Proportion of male *Drosophila* which mated within a 120 minute mating assay exposed to a single Oregon R female, following a 48h exposure to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

3.6.2 Mating latency is not affected by QMP in male *D. melanogaster* with FOXO expression driven in the genital tissues

Mating latency was calculated by finding the difference between the time an individual was introduced to an experiment and the time that mating occurred. This can be considered representative of the willingness and ability of the male to mate with the female, in conjunction with the willingness of the female to accept the male (Fowler et al., 2022).

When comparing across all groups, exposure was found to be a significant predictor of mating latency with an ANOVA ($F(2, 381) = 9.335$, $p = 0.0001102148$). However, when comparing within groups, exposure type was found to significantly affect mating latency only in FOXOxFOXO ($F(2, 66) = 14.62$, $p = 5.55e\text{-}06$) and NOSxFOXO ($F(2, 53) = 4.544$, $p = 0.0151$). Of these two groups, QMP was only found to significantly affect mating latency relative to other exposures in the FOXOxFOXO, reducing mating latency relative to the Starvation-Diet exposure (Figure 25, $p = 0.0029782$) with Ethanol-Diet also significantly reducing mating latency in comparison with Ethanol-Starvation ($p = 0.0000040$). In NOSxFOXO, which has FOXO driven expression in the germ line cells, significant difference in mating latency was not found between QMP-Diet and Ethanol-Diet or Ethanol-Starvation, with Ethanol-Diet found to be significantly lower than the Ethanol-Starvation group ($p = 0.0029782$).

The effect of QMP exposure on germ line cell FOXO expression could not be compared to the parental NOS line due to limited sample size across exposures in this group and so was compared to FOXOxFOXO, which is the maternal parental line. No significant differences were found between FOXOxFOXO and NOSxFOXO in each exposure type ($p > 0.05$) when tested using Tukey post hoc analysis, indicating the the expression of FOXO in the germ line cells was likely not the cause of difference in response to exposure type found in this group as it was also found in the non FOXO expressing FOXOxFOXO line.

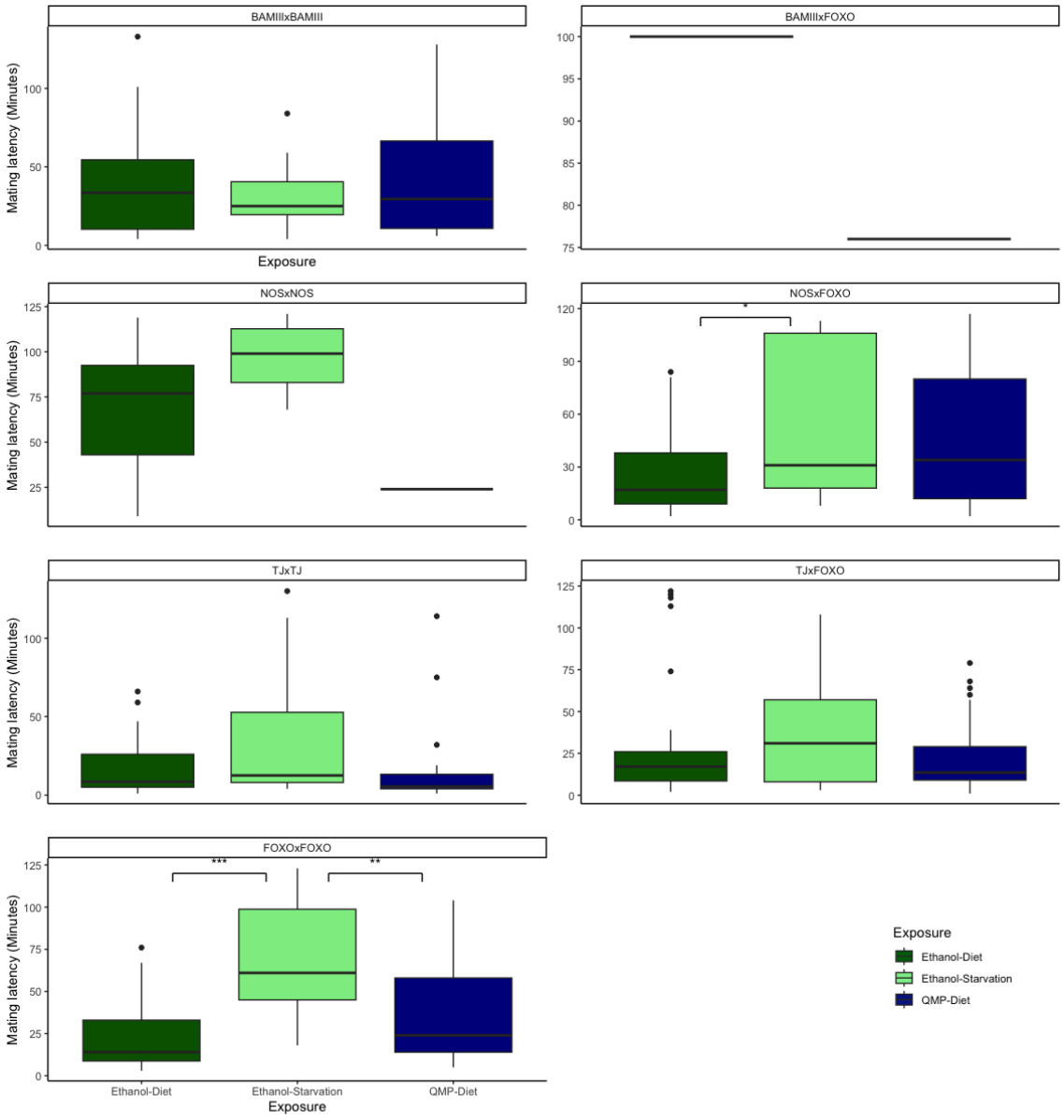


Figure 25. **Mating latency by exposure for each cross in the mating assay.** FOXO crossed line are expressing FOXO in the cell types specified by driver lines, with parental driver line as controls. Sample sizes: BAMxBAM: Diet = 26, Starvation = 22, QMP = 24; BAMxFOXO: Diet = 1, starvation = 1, QMP = 0; NOSxNOS: Diet = 11, starvation = 4, QMP = 1; NOSxFOXO: Diet = 30, starvation = 13, QMP = 13; TJxTJ - Diet: 32, starvation = 28, QMP = 30; TJxFOXO: Diet = 38, starvation = 17, QMP = 34; FOXOxFOXO: Diet = 24, starvation = 24, QMP = 21.

3.6.3 QMP did not affect mating duration in any tested genotype

In previous experiments, QMP exposure has been found not to affect the mating duration of QMP-exposed Oregon R male *D. melanogaster* (Chambers, 2023). Similarly this experiment found restoration of insulin signalling sensitivity in these tissues does not result in QMP impacting the mating duration (Figure 26). As exposure type was not found to impact mating latency when compared across groups by ANOVA, no pairwise testing was conducted. This indicates that restoration of FOXO levels in the late spermatogonia, early spermatocytes, germ line cell and somatic support cells does not impact the mating length in QMP-exposed *Drosophila*.

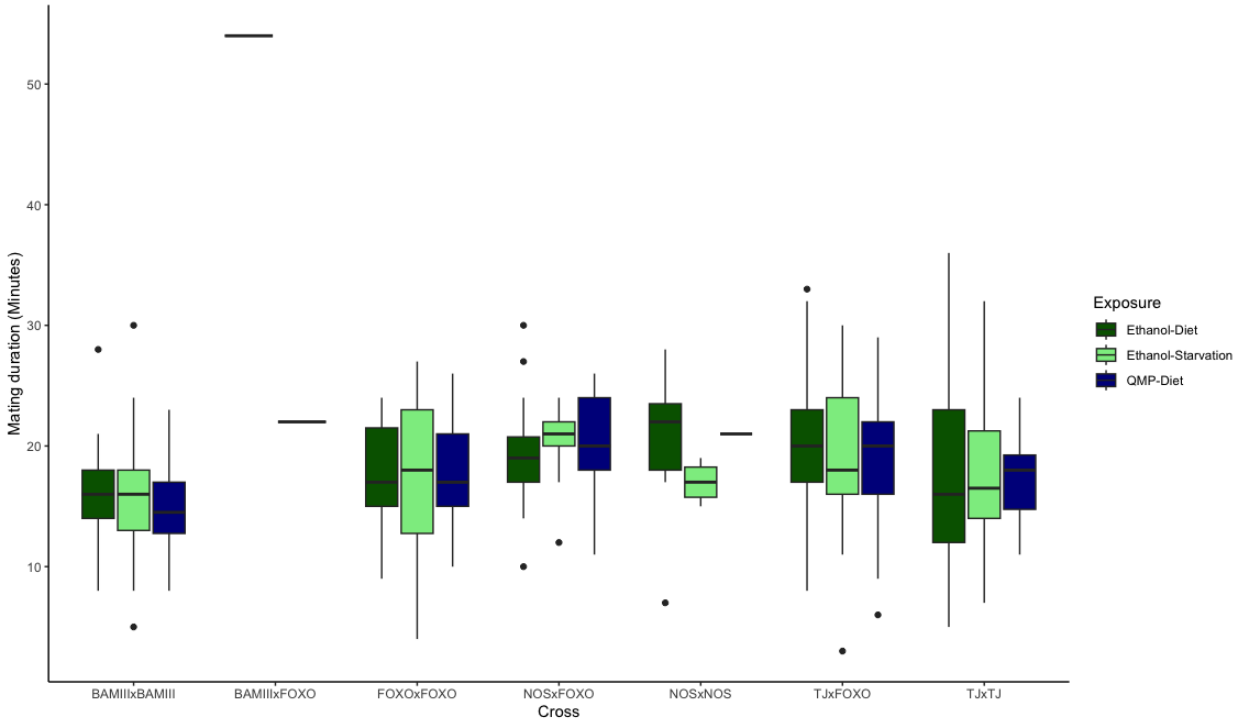


Figure 26. **Exposure type does not affect mating duration in *Drosophila melanogaster*.** Mating duration of male *Drosophila* which mated within a 120 minute mating assay exposed to a single Oregon R female, following a 48h exposure to a liquid diet, starvation or QMP diet exposure. BAMIIIxBAMIII: Diet = 25, Starvation = 21, QMP = 24; BAMIIIxFOXO: Diet = 1, Starvation = 1; FOXOxFOXO: Diet = 23, Starvation = 24, QMP = 21; NOSxFOXO: Diet = 30, Starvation = 13, QMP = 13; NOSxNOS: Diet = 11, Starvation = 4, QMP = 1; TJxFOXO: Diet = 38, Starvation = 17, QMP = 34; TJxTJ: Diet = 32, Starvation = 28, QMP = 20.

3.6.4 QMP did not affect offspring count between exposures in any tested genotypes

QMP has been found not to impact offspring count in male Oregon R *D. melanogaster* (Chambers, 2023), but it has been found to impact ovarian activity in female Oregon R *D. melanogaster* (Camiletti et al., 2016 ; Lovegrove et al., 2020), reducing the number of mature oocytes found within the ovary.

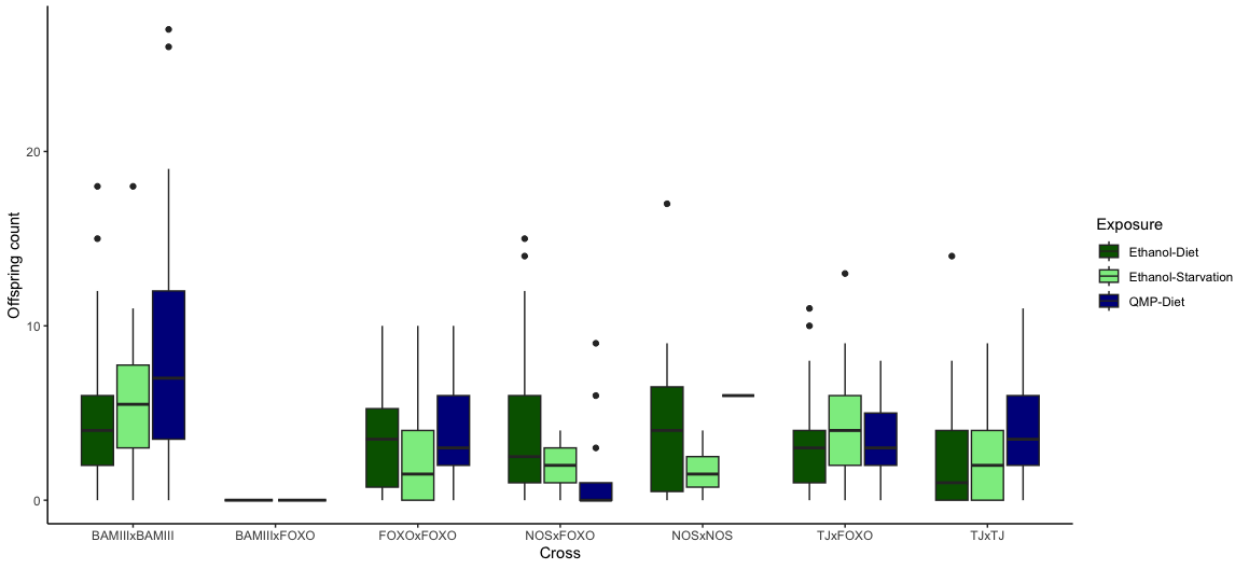


Figure 27. **Offspring count of NOSxFOXO crossed *Drosophila* are sensitive to Exposure type.** The number of offspring produced by male *Drosophila* which successfully mated within a 120 minute mating assay exposed to a single Oregon R female, following a 48h exposure to a liquid diet, starvation or QMP diet exposure. Counts were performed following the eclosuion of the F1 generation and before the first ecclosion of the F2 generation. BAMIIIxBAMIII: Diet = 26, Starvation = 22, QMP = 24; BAMIIIxFOXO: Diet = 1, Starvation = 1; FOXOxFOXO: Diet = 24, Starvation = 24, QMP = 21; NOSxFOXO: Diet = 30, Starvation = 13, QMP = 13; NOSxNOS: Diet = 11, Starvation = 4, QMP = 1; TJxFOXO: Diet = 38, Starvation = 17, QMP = 33; TJxTJ: Diet = 32, Starvation = 28, QMP = 20.

QMP was not found to have significant impact on offspring count in any cross (Figure 27). Only FOXO expression driven in the germline cells found significant effect on offspring count based on exposure type using ANOVA testing on the NOSxFOXO cross ($F(2, 53) = 3.551$, $p = 0.0357$). However, post hoc testing using TukeyHSD found no difference between the exposures.

3.6.5 Post-mating fertility analysis

Post-mating fertility analysis was calculated, based upon the presence or absence of offspring produced by a female 10 days following a successful mating, to allow for all offspring from the first generation to have eclosed. This allows relative infertility to be analysed in each cross rather than the relative level of fertility in offspring producing lines. This is a more powerful analysis of the data than offspring count as it allows for all *Drosophila* mated within the assay to be assigned a fertility status rather than just ones that produced offspring, increasing the sample size in each cross.

3.6.5.1 Effect of QMP on post mating fertility could not be assessed in *D. melanogaster* males with late spermatogonia / early spermatocyte expression of FOXO due to low mating rates (BAM-GAL4 driven)

BAMxFOXO was not able to be statistically compared to BAMxBAM in any exposure due to low numbers of successful matings across all exposures of the BAMxFOXO cross, indicative of very reduced sexual fitness of males with FOXO expression driven in this cell type (Figure 28).

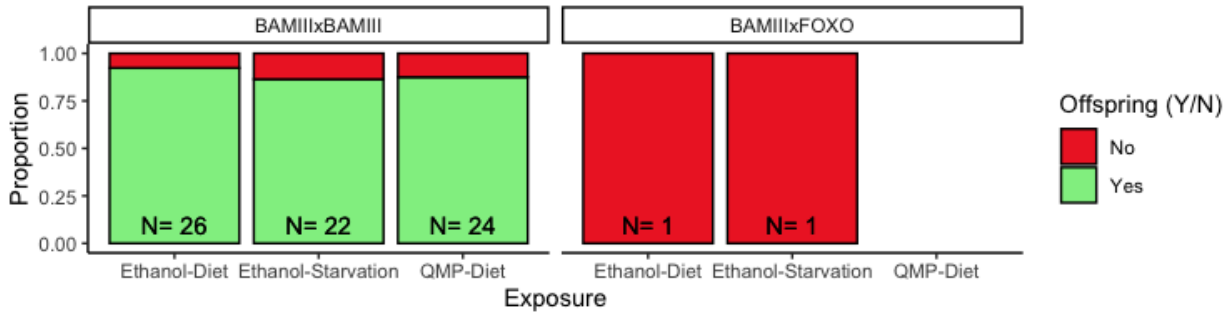


Figure 28. **Proportion of F1 crosses producing offspring following successful mating in BAMxBAM and BAMxFOXO.** The proportion of mated females that produced offspring following having mated with male *Drosophila* within a 120 minute mating assay, following the male having been exposed for 48h to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

3.6.5.2 Germ line cell expression of FOXO results in decreased post mating fertility in QMP exposed *D. melanogaster* males (NOS-GAL4 driven)

NOSxNOS was not found to differ from NOSxFOXO in any exposure group when compared using Fisher's exact test. Post-mating offspring production was found to vary significantly by exposure in NOSxFOXO ($p = 0.002031$) *Drosophila* but not in NOSxNOS.

However, the sample sizes in the starvation and QMP-exposed group of NOSxNOS are likely not large enough to provide processing power to be able to differentiate between the NOSxNOS exposures or against other groups.

When comparing using pairwise Fisher's exact tests, analysis of the NOSxFOXO group found QMP-Diet exposure to significantly decrease offspring production following mating, with reduced chance of producing offspring when compared with both Ethanol-Diet ($p = 0.006721$) and Ethanol-Starvation ($p = 0.003607$) groups. This indicates that QMP exposure reduces the chance of offspring production in *Drosophila* with FOXO expressed in the germ line cells, this effect can not be necessarily attributed to the FOXO expression due to the inability to compare to the parental line, which may also be reproductively repressed when exposed to QMP. Additionally, this impact can not be considered to be indicative of a starvation style response, as reported in females (Lovegrove et al., 2023) as it significantly differs from the starvation-exposed group (Figure 29).

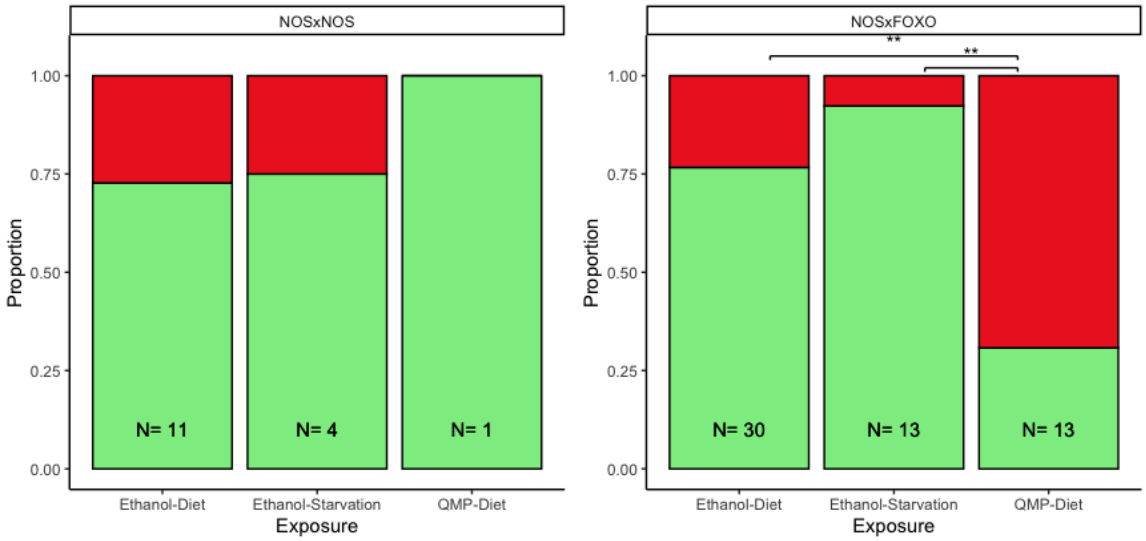


Figure 29. **Proportion of F1 crosses producing offspring following successful matings in NOSxNOS and NOSxFOXO.** The proportion of mated females that produced offspring following having mated with male *Drosophila* within a 120 minute mating assay, following the male having been exposed for 48h to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

3.6.5.3 QMP exposure does not affect post-mating fertility in *D. melanogaster* males with Somatic support cell expression of FOXO (TJ-GAL4 Driven)

Expressing FOXO in the somatic support cells was not found to impact the chance of producing offspring when comparing exposures between TJxFOXO and TJxTJ. No significant differences were found when comparing exposures using Fisher's exact analysis. When comparing exposures within cross types, no significant impact was found on post-mating offspring production in either group (Figure 30).

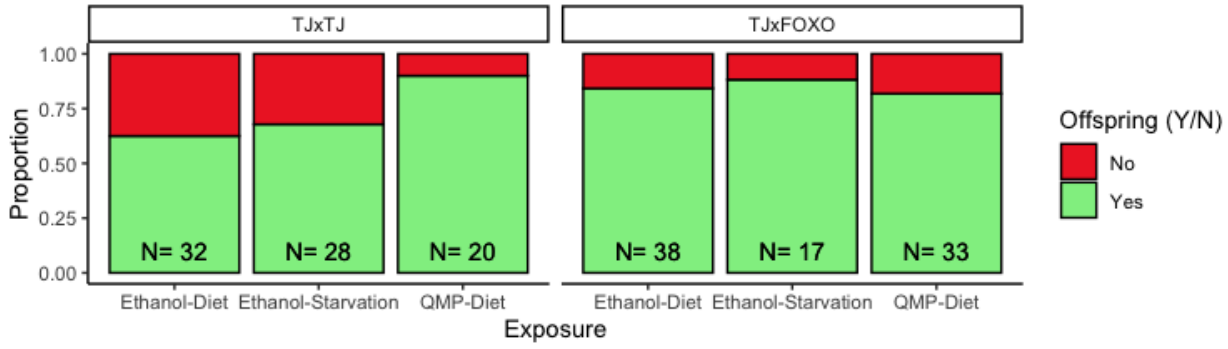


FIgure 30. **Proportion of F1 crosses producing offspring following successful mating in TJxTJ and TJxFOXO.** The proportion of mated females that produced offspring following having mated with male *Drosophila* within a 120 minute mating assay, following the male having been exposed for 48h to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

3.6.5.4 QMP increased post mating fertility in FOXOxFOXO control line *D. melanogaster* males compared to starvation exposed males

When assessing the chance of offspring production between exposure types in the FOXO parental line (y[1] w[*]; P{w[+mC]=UAS-foxo.P}2) using Fisher's exact tests significant impact was found (p = 0.0426). QMP exposure was found to significantly increase the chance of offspring production following mating in comparison to Ethanol-Starvation (p = 0.02475), but not with Ethanol-Diet (p = 0.1556416) (Figure 31).

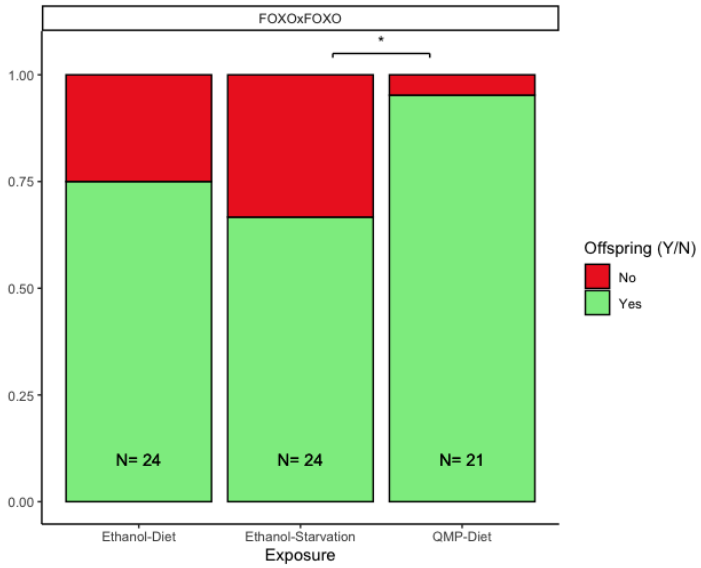


Figure 31. **Pairwise analysis of proportion of F1 crosses producing offspring following successful mating across exposures in each group type in FOXOxFOXO.** The proportion of mated females that produced offspring following having mated with male *Drosophila* within a 120 minute mating assay, following the male having been exposed for 48h to a liquid diet, starvation or QMP diet exposure. N for each exposure and genotype combination found within each respective box of the graph.

Section 4 - Discussion

QMP represses reproduction in worker honeybees (Winston and Slessor, 1992) but acts as a sex pheromone in drones (Gary, 1962; Gary and Marston, 1971; Brandstaetter et al., 2014). The basis of this sex-specific difference is not understood. Despite the substantial evolutionary distance between honeybees and *Drosophila*, *Drosophila* females have also been found to be reproductively repressed by QMP through the induction of starvation-like response (Lovegrove et al., 2023). Equivalent research of QMP exposure on males is limited, however they have been shown to not have their reproductive capacity reduced by QMP (Chambers., 2023). This study aimed to further investigate the effect of QMP on *D. melanogaster* males, to determine the extent to which QMP has similarly dimorphic effects as is found in honeybees and whether insulin signalling in the testes is responsible for any dimorphic effects.

4.1 Responses of male *Drosophila* to QMP

4.1.1 QMP increases feeding during exposure

When investigating post-exposure feeding levels in male *Drosophila*, QMP was not found to impact the level of food intake (Figure 10, Figure 13), contrasting with findings in females (Lovegrove et al., 2023), where QMP exposure of the same length led to increased feeding levels using the same feeding assay style. This indicates a dichotomy of response to QMP exposure between male and female *Drosophila*. However, during the experiment, it was noted that the abdomen of QMP-exposed males was frequently ‘milky’ in colour. Dissection of a sample of these *Drosophila* found large white-coloured crops, the *Drosophila* food storage organ. The high prevalence of this phenotype in QMP-exposed males and the lack of significantly different feeding post-exposure was considered potentially indicative of increased feeding during the exposure and so further feeding assays were conducted to quantify the level of food consumed within the exposure period.

Intra-exposure feeding levels were influenced by QMP exposure, with QMP-exposed *Drosophila* consuming significantly more diet than both liquid diet and starvation-exposed

individuals. However, as the feeding analysis took place during the exposure period, intra-exposure feeding was measured in modified falcon tubes over the 48 hour exposure period rather than on agar following exposure as in the post-exposure feeding assay which may have resulted in some slight feeding differences between the experiments, although this difference would be standardised between exposures in experiment. This seems to indicate that sex-based differences in post-QMP exposure feeding in *Drosophila* are due to an inability to ingest further food due to crop fullness. If the male has consumed food sufficient to fill its digestive system,there might be no scope to ingest further food which might be indicative of a lack of ability to feed rather than a lack of drive to feed. It is possible that sex-based differences contribute to a dichotomy in this instance, as female *Drosophila* are known to feed more than males (Wong et al., 2009). This is hypothesised to be due to higher levels of nutritional investment associated with gamete production (Piper and Partridge, 2017). Assuming that reduced capacity for food intake is responsible for the reduced feeding, it is possible that females have a higher through-rate of food, meaning that they are less likely to become limited as to the quantity of food they can intake. This could be tested by evaluating the relative transit time of each sex by collecting male and female *Drosophila*, aging them for 24 hours, briefly allowing them to feed on dyed food and then measuring the first point at which dye appears in their excrement.

However, there are other potential explanations for the dichotomy found in QMP-induced feeding between male and female *Drosophila*. Whilst the low level of post-exposure feeding seems to be due to a lack of ability to eat, it is also possibly due to a lack of drive to eat, as despite a lack of dye intake post-exposure being indicative of low food intake, it does not necessarily correlate with low ability to eat. If the differentiation between post-exposure feeding in males and females is due to a lack of drive to eat, for example if the drive to eat is low. Low feeding levels could be indicative of the signalling induced by QMP persisting for longer in females than in males, for example differing insulin signalling over time. This line of thought assumes that females are also induced to feed during exposure and that the effect lasts longer in females than in males, but that is not necessarily true and would need to be further researched.

Whilst it is plausible that female *Drosophila* experience induced feeding during exposure period, this is not categorically known and further research in this area would be useful. For example, a possible explanation for the difference in post-exposure feeding drive

between males and females could be that QMP induces a sex-specific relative nutritional deficit in females, through the reduction of feeding during the course of exposure, which could theoretically result in a period of compensatory post-exposure overfeeding, this is possible given that a following starvation an increase in feeding is known to occur (Sudhakar et al., 2020).

To distinguish between a lack of drive to eat and a lack of ability to eat in males and to compare the drive to eat following exposure to QMP between male and female *Drosophila*, a proboscis extension assay could be conducted using methodology modified from Wong et al. (2009). This would allow assessment of feeding motivation and a calculation of food intake per proboscis extension, which would serve as a metric of ability to consume food.

Whilst the feeding assay setup is a good indicator of feeding, in the starvation and diet exposures of intra-exposure testing dye intake was not found to differ significantly. Initially this seems surprising as dye intake was used as a proxy for food intake. However, given that the starvation exposure contains no food, the dye intake in this exposure must be due to non-feeding activity such as drinking. This reduces the ability of these results to be considered representative of just food intake, as the drive to consume food can not be separated from the drive to drink. Therefore, it is possible that QMP induces an increase in drinking rather than food intake. This is a difficult distinction to elucidate because adult *Drosophila* consume a liquid diet (Laplante and Sabatini, 2009) and so any attempts to feed them with solid food are no longer representative of natural feeding behaviour. In order to calculate relative food intake as distinct from drinking, either dyed solid food (which would be a non-natural feeding method) could be used in the feeding assays, or feeding assays with the choice of liquid and starvation diets with a second dye colour, could be conducted. This would allow the calculation of relative preference of liquid food relative to non food containing liquid intake.

4.1.2 QMP exposure results in distinct abdomen phenotype

Potentially a phenotypic clue to the feeding difference

In order to quantify the prevalence of the 'milky abdomens' frequently observed in the QMP-exposed group relative to non-exposed individuals, a semi-quantitative scale was developed. QMP-exposure was found to significantly impact the phenotype of the abdomen, with more stage 2 'milky' abdomens found in the QMP-exposed group compared to non QMP-exposed flies. Dissection of flies with stage 2 abdomens found crops that were cloudy and large in size when compared to stage 1 (Figure 14). As crops are storage organs within the *Drosophila* digestive system, the stage 2 phenotype is likely to be indicative of high levels of feeding. Wong et al. (2008) noted that the abdomens of nutritionally-restricted *Drosophila* that were given access to food had abdomens 45% larger that non-restricted individuals with access to food. This is very similar to the phenotypes prevalent in QMP-exposed male *Drosophila*, suggesting that the stage 2 phenotypes seen in this group might be the result of induced starvation in the presence of food.

This supports the hypothesis that the post-exposure difference between males and females is due to a lack of drive to feed as a starvation effect may no longer be being induced, but it might also be a result of a lack of ability to eat due to the crop being full. Alternatively, the phenotypic difference could be due to altered signalling within the crop itself, but this seems unlikely as the optical density analysis independently supported the theory that increased feeding took place during the exposure period.

It is natural to assume that stage 2 crops are indicative of higher levels of food within the crop as they are found more frequently in a group with higher intra-exposure food intake. However, the food intake scoring used in this study was based upon total dye within the whole *Drosophila* rather than specifically within the crop. As a result, whilst groups with milky abdomens correlate with groups with increased total food intake, this does not necessarily mean that the milky abdomen is representative of a crop more full of food. Repeating this experiment with a QMP-Starvation exposure group would allow greater understanding as to whether the stage 2 phenotype is due to QMP exposure directly or mediated through an increase in feeding. If the QMP-Starvation group does not have a

similar level of ‘stage 2’ abdomens, it is likely that the increase in the prevalence of these abdomens in the QMP-Diet group is exclusively based on increased feeding. Alternatively, if ‘stage 2’ abdomens are induced directly due the action of QMP on the crop, the proportion of ‘stage 2’ abdomens would be similar to that seen in the QMP-Diet group. However, it seems likely, based on the low levels of stage 2 in the Ethanol-Starvation group relative to the Ethanol-Diet group, that the milky phenotype is likely due to food consumption differences rather than QMP directly impacting the phenotype. Additionally, the quantity of food in the crop could be assessed by conducting an intra-exposure feeding assay followed by dissection, removing the crop and performing optical density analysis. This would allow for comprehensive quantification of dye in the crop following the exposure. To specifically test the hypothesis that the lack of food intake post-exposure is due to a lack of ability to feed, sequential feeding assays could be performed using dyed foods with different absorbance wavelengths for the intra-exposure and post-exposure phases of the experiment. The relative absorbance at each wavelength following optical density analysis would allow quantification of feeding at each stage. Analysing the results in conjunction with the proboscis activity assay suggested above would elucidate whether the difference in feeding activity intra-exposure and post-exposure is due to a lack of ability to eat more or a lack of drive to eat following exposure.

4.1.3 QMP reduces activity in male *D. melanogaster*

QMP exposure increased the feeding activity of male *Drosophila*. It has also been found to increase the feeding activity of female *Drosophila* and induce a starvation-like insulin signalling effect (Lovegrove, 2023). As a result, it seems logical to assume that QMP is consistent with this in males, and again induces a starvation-style response. In *Drosophila,* the level of starvation is known to affect the level of locomotive activity (Bell et al., 1985 ; Yu et al., 2016). As such, quantifying the level of activity change associated with QMP exposure could allow classification of the induced result as either starvation-like or non starvation-like. Current understanding on the effect of QMP on male *Drosophila* activity is limited. Croft et al., (2017) found that males orient preferentially towards QMP in a t-maze test compared to an ethanol control, indicating that QMP exposure impacts spatial distribution of male *Drosophila* which may therefore suggest an impact on activity. However, these results are not directly relevant to locomotion as it tests the attraction to

QMP in an acute setting rather than after a period of exposure. As such, understanding of the absolute impact of QMP on male *Drosophila* locomotive activity is not known.

QMP significantly reduced the activity of male *Drosophila* when compared to both diet and starvation groups, across both the day and the nighttime. This indicates that the activity induced by QMP can not be considered to be representative of a starvation style effect, at least at the levels naturally experienced by a *Drosophila* male in the first 24 hours of true starvation. However, the relationship between food and level of locomotion is complex in *Drosophila*. Starved flies typically increase locomotion over the period of starvation until food is eaten or they are in the presence of food (Yu et al., 2016). This means that, if QMP is inducing a starvation effect in the presence of food, then the exposure to the food and any subsequent consumption is likely to reduce the level of observed locomotion even if a sustained state of starvation has been induced. It is therefore possible that the decrease in activity seen in male *Drosophila* in the present study was impacted by food intake and exposure, and that QMP can not be considered to decrease activity on its own, but rather by acting in conjunction with access to food. In order to test the extent to which this occurs, the impact of QMP exposure without an increase in food intake could be investigated by measuring the activity of QMP-Starvation exposed *Drosophila*.

A limitation of this experimental setup was that low activity levels are not necessarily indicative of low movement because movement that is confined to one area of the tube will not result in an increase in recorded activity if the beam is not broken. Whilst one beam was positioned close to the point of exposure, it could not be directly over it because movement of the filter paper during the exposure duration could have potentially obscured the beam. Furthermore, activity is only monitored in the horizontal plane, meaning that movement over the exposure-containing filter paper in the vertical plane would not result in increased recorded activity. Additionally, the results of the current experiment give no indication as to the point at which the fly is located other than the relative activity levels found at each ring of sensor beams. Therefore, the exact position of the flies are not known over the course of the experiment and it is possible that QMP is inducing increased localosed movement, resulting in exploration of less of the tube resulting in less activity recorded, without reducing total movement by the *Drosophila*. This could not have been easily checked in the current setup because opening the enclosure to visualise the position would have disturbed the *Drosophila*, altering their activity.

Camera-based tracking of position over time could improve understanding of exact positions within the tube whilst also being able to give greater insights as to the movement patterns in each group. An example of such a setup is the EasyFlyTracker system as described by Qu et al. (2022).

4.1.4 QMP reduces survival rate of male *D. melanogaster*

QMP exposure significantly reduced survival rate 48 hours post-exposure, compared to both diet and starvation exposures.

No studies have been conducted directly assessing the effect of QMP on lifespan. However a marginal increase in mortality has been found when exposing *A.mellifera* workers to QMP in caged experiments (Anthony Bracuti., Personal communication). In *Drosophila,* insulin signalling is known to dramatically alter lifespan. *Chico* is a key protein in insulin-like protein reception. Loss-of-function mutant *Drosophila* for this protein, which have reduced capacity to respond to complete insulin signalling, live 36% or 48% longer in their heterozygous or homozygous states respectively (Clancy et al., 2001). As a result, increased insulin signalling is thought to contribute to factors which accelerate the ageing process. If QMP alters insulin signalling in males in the same way that it does in females (Lovegrove et al., 2023), it is possible that QMP induces excess insulin signalling that, in turn, might lead to the early aging of *Drosophila*. However, it is unlikely that this would serve as the sole explanation for the increased mortality at 48 hours post-exposure due to how much shorter this is than the lifespan of the *Drosophila*, leading to the increase in mortality seeming to be more likely to be due to toxicity than accelerated aging. Whilst the increase in mortality could seem a reasonable explanation for the reduction in activity in the QMP-exposed group, no instances of death were recorded at 24 hours post-exposure in the activity assays meaning this could not be a factor.

A logical way of testing whether QMP increases insulin signalling in male *Drosophila* would be to test the expression level of products of the insulin signalling system in a range of tissues in QMP-exposed and non QMP-exposed males, as seen conducted on females by Lovegrove et al., (2023).

When Chambers., (2023) studied the lifespan of male flies exposed to equivalent diet and starvation exposures, high rates of survival were found until 48 hours, at which stage there was a rapid increase in mortality, which was anecdotal attributed to the lack of moisture found on the filter paper in the exposure tubes at this stage. However, desiccation is unlikely to explain the higher mortality rate in the QMP group in the current experiment as filter paper was found to still be damp at the point of recording mortality. Furthermore, given the higher viscosity of the QMP exposures relative to the liquid and starvation exposures, it is likely that the other exposures would face desiccation first if that were to be a limiting factor. It is, however, possible that the higher viscosity increased the mortality rate in situations in the QMP-exposure group due to more pronounced blocking of spiracles in situations where the body of the fly came into direct contact with the exposure leading to coating.

4.1.5 The effect of QMP on insulin signalling in the testis

Given that QMP exposure in male *Drosophila* has been found to induce feeding similar to that found in female *Drosophila* (Lovegrove., 2023) and that the resultant reproductive repression in females has been found to be caused by altered insulin signalling in the female genitals (Lovegrove., 2023), it seems logical to assume that similar insulin signalling might occur in the male genitals. To try to quantify the level of insulin signalling in the testes, a TGPH4 line was used (Methodology in supplementary section 6.2). This line ubiquitously expresses a protein comprising two parts: a PIP3 localiser and a Green Fluorescent Protein. As the reception of insulin-like peptides during insulin signalling leads to the addition of a phosphate group to PIP2, creating PIP3 which are localised to the cell membranes, increased insulin signalling should lead to increased localisation within the cell towards the cell walls, result in a fluorescent band around the edge of cells. In contrast, in cells with low insulin signalling, the low levels of PIP3 should result in fluorescence spread across the cell structure (Britton et al., 2002). The TGPH4 line was theoretically promising for assessing insulin signalling differences between exposures without the need for more complex genetic expression analysis such as RT-qPCR as well as allowing for cell-specific information on insulin signalling which can not be found through RT-qPCR.

In practice, differentiation of starvation and diet-exposed male *Drosophila* testes was not possible using this line. This is possibly because *Drosophila* testes are insulin insensitive

(Tang et al., 2011) and therefore differing levels of insulin signalling are not induced by starvation. However, given that the insensitivity in the testes is considered to be mediated by low *FOXO* levels, which acts further into the insulin signalling system than the PIP2 to PIP3 conversion, this reduction in insulin sensitivity does not imply that the cells can not display banding in the presence of insulin like peptide reception (Figure 4). As such, the banding should be seen even in cells that are rendered insensitive due to low *FOXO* levels. Ultimately, due to the inability to differentiate between diet and starvation-exposed *Drosophila*, this method was not a feasible way to understand if QMP induced a starvation-like effect on insulin signalling. In order to gain understanding of the impact of QMP exposure on insulin signalling in the testes, RT-qPCR could be conducted on components of the insulin signalling pathway following QMP exposure, or sensitivity of the testes to insulin signalling could be restored and the impact of QMP on aspects of sexual fitness could be subsequently analysed. This latter option was pursued in the current study.

4.1.6 Mediation of QMP response by FOXO levels in the testes.

The starvation-like effects of QMP have been found to reduce the reproductive capacity of *Drosophila* females (Lovegrove, 2023). However, the current experiment indicates that similar starvation-like effects are induced in males without reducing reproductive capacity (Chambers., 2023). The differential impact of induced starvation style response is likely a result of either, different insulin signalling in the genital tissues between sexes, which could be analysed using qPCR, or a difference in reproductive capacity following the change of insulin signalling in these tissues.

Drosophila genitalia are known to be insulin insensitive due to low FOXO expression levels in the developing genital tissues (Tang et al., 2011). As the insulin signalling pathway acts through *FOXO* by repressing it, if the levels of *FOXO* are already very low, the testes can be considered to be in a permanent state of FOXO repression and as such, further repression of FOXO by insulin signalling can be considered to have negligible action on cellular function. This makes the genital tissues less sensitive to insulin signalling through this pathway, leading to non-nutritionally plastic development (Tang et al., 2011). This supports the idea that insulin signalling may be similarly affected in male *Drosophila* testes as in female ovaries but that it does not result in differing sexual fitness.

Developmental nutritional plasticity has been found to be restored in the genitals of male *Drosophila* through the use of GAL4/UAS driven expression of FOXO in the genital imaginal discs (Tang et al., 2011) and the posterior lobe of the genital arch (Dreyer and Shingleton, 2019), reducing the genital size in both experiments, with subsequent sexual fitness metrics reduced in both cases. As such, both tissues can be considered to be naturally insulin insensitive, allowing for prioritisation of development in the genital tissues in the developing male testes.

These experiments focused on the impact of FOXO-mediated sensitivity during development and found significant impact on subsequent tissue development. As sperm are generated throughout the life of the adult male *Drosophila,* and are critical in reproductive success, they might be protected from nutritionally sensitive growth as a result of their reduced ability to respond to insulin signalling, similar to other genital tissues. Given this, we hypothesise that restoring *FOXO* to tissues within the testes cell types related to sperm production, rendering them sensitive to insulin signalling, might allow them to be repressed by QMP, which in turn might reduce a range of sexual fitness metrics.

4.1.6.1 Mating success

Dreyer and Shingleton (2019) found mating success was reduced in male *Drosophila* with FOXO expressed in the posterior genital arch during adult genital tissue development. This was largely attributed to reduced genital size in these crosses. Whilst the genital size in the crosses we used did not obviously differ in size from that of wild type *Drosophila*, it is possible that the expression of FOXO in tissues in the testes during development of the adult tissues resulted in differing genital structures that are noticeable to prospective mating females, resulting in differing levels of reception by the female for mating. Furthermore, it is possible that the mating willingness of the male is impacted by sperm quality, which would result in mating latency, which is impacted by the level of sperm development occurring. Whilst there is little direct evidence of this in the literature, males are known to alter ejaculate size based on the perceived fitness of the female (Lupold et al., 2010) and so it is not infeasible that this could occur. However, this proposition seems unlikely as it would not be in the best interest of the individual to reduce mating efforts in wildtype flies, as the inherent lack of nutritional plasticity in the male genitals means that sperm production is unlikely to be the main factor preventing offspring production by

males. The limiting factors are more likely to be access to mates and post-mating sperm selection by the female. Female reception to male-attempted mating is impacted by various proxies of development that are considered to be representative of likely sperm quality. One such example is in courtship song selection, with more energy-intensive songs preferred by the females (Talyn and Dowse, 2004). If a male has little energy to dedicate to courting and therefore is unable to attract females, it is likely that access to mating will be the limiting factor in offspring production regardless of the quality of sperm.

When comparing the mating success between crosses, only two groups were found to have exposure type significantly impacting on mating success, TJxTJ and TJxFOXO. All other crosses found no impact of QMP exposure on mating success, which is consistent with results found in the wildtype *Drosophila* Oregon R (Chambers, 2023). In the non FOXO-expressing TJxTJ parental line, QMP exposure reduced mating success compared to both diet and starvation exposures. The difference in exposure type on mating success in the TJxTJ line is unexpected as it shows impact of QMP exposure despite no FOXO expression in this line, thereby meaning that there should be no nutritional plasticity expected in this line. Given that only lines containing TJ backgrounds were found to have a significant effect of QMP relative to other exposure types, It is possible that a component of the TJ genetic background facilitates QMP sensitivity. This could potentially be due to differential insulin signalling in this line. In order to assess whether this is the case, the genetic backgrounds of the line could be standardised so that the only differences in genetics are the regions specifying the expression of GAL4. This standardisation of the genetic background would allow better isolation of the impact of the GAL4 driver whilst minimising non-driver differences and could be conducted through repeated backcrossing of driver lines to Oregon R, in order to minimise any impact on the results due to genetic variance.

The lack of significance between the diet and starvation exposures in the TJxTJ line suggests that the QMP-induced difference is not due to an induced starvation effect, but rather, is more likely to be induced through other means. However, these other means remain unknown. It is possible that the factors which increase the rate of mortality seen in QMP-exposed male *Drosophila* contribute to this. Such factors might include metabolic stress, but the specific factors impacting mortality and therefore possible impacting mating

success also remain unknown at this stage and, even if these factors do cause the change in mating success, it is unclear why this would occur specifically in lines containing the genetics of the TJ driver.

In the TJxFOXO cross, which expresses FOXO in the somatic support cells, QMP exposure increased mating success in comparison to starvation, with no significant difference found between QMP-exposed and diet. This indicates that starvation itself reduced mating success but that QMP exposure did not, suggesting that a starvation-like response is not found in the QMP group, due to the significantly higher proportion of mating success found in the QMP-exposed group than the starvation group.

Post-mating sexual fitness metrics were difficult to assess as a result of limited sample sizes in groups that had low levels of mating success, for example in BAMxFOXO where low mating success resulted in inadequate sample size for statistical comparison. As a result, comparative analysis for BAMxFOXO and NOSxNOS can not be comprehensively performed, with low significance unavoidable due to low through rate of these lines. In order to address this issue, in future experiments, males could be left with the females following the mating assay for 24h, with both being removed at that stage, leading to a larger opportunity for mating to occur in lines which did not readily mate in the mating assay. The NOSxNOS line also failed multiple times during the experimental period leading to difficulties in sample size for this cross.

4.1.6.2 Mating latency

Like mating success, in *Drosophila* mating latency is impacted by the perceived fitness of the male courting the female, with male *Drosophila* with ‘short genitals’ found to have marginally longer mating latencies than those with ‘long genitals’ (Dreyer and Shingleton, 2019). Furthermore, in female *Drosophila*, starvation results in reduced copulation rates and increased mating latency (Sun et al., 2023) and, in males, starvation results in feeding being prioritised over mating (Ando et al., 2020). This means that any variance in mating latency could potentially be attributed to a number of factors. Firstly, if QMP is inducing a starvation-style effect strong enough to create the feeling of hunger, it may result in feeding being performed preferentially to the attempt to mate with the female. This would

lead to longer mating latency times. However, this is unlikely as it was not found in Oregon R *Drosophila* (Chambers, 2023) and the restored insulin signalling sensitivity should be within the testes and so would not be hypothesised to directly impact the drive for the feeding. Secondly, it could be due to abnormal development of the genitals in the QMP-exposed FOXO expressing lines, as abnormal genital structure is known to impact the mating success of males as already described, and would likely lead to lower drive of the females to mate with these males. Thirdly, if the restored insulin sensitivity in the testes has resulted in lower quality sperm production and that it is a factor that modifies male drive to mate, it is possible this would impact mating latency.

In Oregon R *Drosophila* males, QMP has been found to result in mating latency that is significantly shorter than that of starvation groups but not diet-exposed groups (Chambers, 2023). Similarly, Croft et al. (2017) found no significant difference when comparing diet and QMP exposure on a subset of mating latency defined as ‘low mating intensity’, which consisted of pre-orientation, orientation to a female and tapping behaviour. In contrast to the findings of Chambers. (2023), only two groups were found to be sensitive to exposure type NOSxFOXO and FOXOxFOXO, leaving limited capacity for QMP-exposed groups to be considered to induce a response which could be defined as either starvation-like or diet-like. In both lines, starvation resulted in significantly increased mating latency in comparison to diet. However, in the NOSxFOXO line, QMP was not found to differ significantly from either exposure, meaning the effect can not be classified as starvation-like. In the FOXOxFOXO line, consistent with the findings of Chambers (2023), QMP exposure resulted in significantly lower mating latency than starvation exposure *Drosophila* with no significant difference from diet exposure. The results suggest that targeted FOXO expression in the germ line might induce a more starvation-like style response than the FOXOxFOXO parental line, but there is large phenotypic difference between the NOSxFOXO and FOXOxFOXO line which may lead to differential mating reception by the female. As a result, this may lead to the same level of mating effort from the male resulting in varying levels of success between genotypes.

4.1.6.3 Post-mating success

The aim of this mating assay was to express FOXO in the testes in order to see, firstly, if this renders sperm developmentally sensitive to insulin signalling and, secondly, to see if

QMP-induced fitness metrics in these lines deviate from those of non FOXO-expressing *Drosophila*. Given that restoration of insulin sensitivity has been shown to impact the development of cells (Tang et al., 2011) and that sperm are developed during the adult stage of male *Drosophila* (Demarco et al., 2014 ; Fabian and Brill, 2012 ; Fuller, 1998), it seems that the performance of the sperm would most likely be impacted by QMP in *Drosophila* expressing FOXO in tissues related to sperm development, which would be likely to impact the post-mating success as they are metrics based on the ability of the sperm to fertilise the female *Drosophila*. However, other factors may impact post-mating success, such as the ability to transfer sperm to the female.

Offspring number has historically not been found to be affected by QMP exposure or starvation (Chambers, 2023). Assuming that QMP acts through impacting insulin signalling in the genitals and that the genitals of male *Drosophila* are naturally insensitive, this is what may be expected. However, consistent with these findings, QMP was not found to impact the number of offspring produced in each group. The ability to find significant differences in this instance was limited by the low sample size, determined by both the number of successful mating events in the mating assay and also by the high prevalence of mated females not producing any offspring. This means that the low significance between groups can not be definitively considered reflective of no difference in fertility between groups.

In order to increase statistical power, a more robust mating success metric in this instance would be to compare successful to unsuccessful offspring production following mating. This was not something that was undertaken by Chambers (2023). Only two groups were found to have offspring production success that was sensitive to exposure type, NOSxFOXO and FOXOxFOXO. In FOXOxFOXO crosses, which have no driver of FOXO expression, QMP exposure was found to increase offspring generation probability following a successful mating in comparison to starvation. However, in NOSxFOXO crosses, QMP exposure reduced the probability of offspring production relative to diet and starvation exposure. This indicates that the restoration of FOXO levels in the germ line cells results in a reversing of the impact of QMP on the fertility of males.

The effect of QMP on the probability of offspring production in NOSxFOXO indicates that the resistance of male *Drosophila* testes to changes in fertility following QMP exposure

may be due to low *FOXO* levels in the germ cells, rendering them insensitive to insulin signalling and potentially indicating that the germ cells may mediate the dichotomy of sex based QMP response, with female genitals sensitive to insulin signalling and male genitals not. Increased *FOXO* levels in these cells can lead to fertility mediated by QMP, explaining the low levels of offspring production following mating in the NOSxFOXO QMP-exposed group. If QMP was producing this effect due to inducing a starvation like effect in these cells, we would expect to find starvation exposed flies also differing significantly from the diet also experiencing low post-mating success due to similarly raised insulin signalling based on starvation, which is not seen. This could be explained by threshold: if QMP reduces insulin signalling such that Akt expression is lower than that seen in starved individuals then the magnitude of the effect could be large in QMP-exposed individuals but not in starved individuals.

In the FOXOxFOXO line, the increase in offspring production following QMP exposure is surprising given that this line contains no driver, meaning that it would not be expected to express FOXO in any way that deviates from natural expression. Therefore, it would be predicted that this line would act similarly to the other parental lines that do not express *FOXO* in the genital tissues, which see no significant impact of QMP exposure on post-mating offspring production. However, it is possible for UAS lines to have leaky expression, leading to non-natural FOXO expression that is not controlled by the GAL4 driver in the FOXOxFOXO line (Akmammedov et al., 2017). This leaky expression would not be specifically localised to the testes and may even occur during the developmental stages, which are known to have an impact on the sexual fitness of the adult *Drosophila* (Tang et al., 2011). If leaky expression is found within the line, it would still result in genitals that are insensitive to insulin signalling unless it is found within the specific cells that restrict this signal transmission. However, Insulin signalling is a system that has multiple action pathways stemming from Akt and so the removal of the ability to repress *FOXO* does not mean that insulin signalling has no way of impacting the cells. It is possible that the level of activation of the differing action pathways mediates the fertility of male *Drosophila,* resulting in differing effects of QMP exposure between lines, which may be the case if leaky expression is found in other cells. If this is the case it would point to a more complex reception of QMP than just insensitivity based on lacking FOXO expression during the reception.

In both crosses with QMP-specific responses, QMP significantly differed from the starvation exposure. Furthermore, in both cases, diet and starvation did not significantly differ from one another. As such, QMP can not be considered to be inducing a starvation response in this instance as QMP exposure significantly differed from starvation exposure. These findings indicate that males are not impacted by QMP in the starvation-like way that has been hypothesised in female *Drosophila* (Lovegrove et al., 2023).

One important consideration when comparing the impact of exposures on fitness using offspring based metrics is that an assumption is being made that the impact of sperm quality would be large enough to result in reduced offspring production. In reality, reduced sperm quality or number does not necessarily mean reduced fertility, especially in an instance with no sperm competition against other mating males (Garbaczewska et al., 2012). The metrics used for assessing fertility in female *Drosophila* are the number of mature oocytes, which act as a proxy for fertility without actually performing mating assays and quantifying the number of offspring. In females, this is a sensible metric as, following a mating event, there are many sperm competing to fertilise a single egg, meaning that a reduction in number of mature eggs will likely result in a reduction in the theoretical offspring able to be produced following a mating event. However, this is not likely to be the case in males. With many sperm competing for one egg, it is likely that sperm count is largely redundant, so that sperm number reduction does not necessarily result in less offspring produced as long as each egg is fertilised. In order to assess the impact on sperm fitness, which would be an equivalent metric to the number of mature oocytes, a number of tests could be performed. Analysis of sperm density and motility would allow for analysis of the relative fitness of the sperm (Garbaczewska et al., 2012 ; Jamie Smith., personal communication, University of Hull). This would be a powerful metric as it would allow for levels of change in sperm quality that may not result in differing levels of offspring production. Further mating assays with a control male in the same environment would allow for sperm competition analysis, with female *Drosophila* able to select which sperm to use based on aspects of sexual fitness of the male (Fiumera et al., 2004). By mating a female with both males and looking at the proportion of the offspring from each partiline, a relative fitness of the sperm could be calculated, allowing more comprehensive analysis of sperm quality in a real life setting.

4.1.6.4 Mating assay limitations

If there is not sufficient successful mating in a particular line then it is not possible to statistically analyse mating outcomes that are dependent upon data from that line. This was the case for BAMxFOXO which had very low rates of mating. This prevents statistical analysis in post-mating sexual fitness metrics. However, it is still indicative of the substantial impact of FOXO expression in the developing sperm on sexual fitness of male *Drosophila*. This was also particularly problematic for the NOSxNOS line, which failed multiple times throughout the experiment and had low successful mating rates. As a result, the impact of QMP on NOSxNOS is not possible to assess and the ability to perform comparative analysis of this line to others is limited.

Whilst GAL4/UAS lines can be used to quickly express genes of interest in specific tissues, any difference in effect can not be specifically attributed to the expression unless the lines are otherwise genetically identical. Variations in response may be due to other genetic variance between the lines outside of the expression of GAL4. Examples of other genetic differences impacting mating success might include phenotypic differences. For example, the FOXOxFOXO line is yellow in colour, which is not the case in any other line. Whilst it is not intuitively obvious why this would have any impact on the effect of QMP, it could affect the mating reception of males from different lines by the female, leading to different mating behaviour. In order to account for this, backcrossing of the lines used with Oregon R would reduce genetic variation between lines outside of the UAS or GAL4 specifying regions, leading to less chance of this variation affecting results. This would involve successive crossing of driver lines with a known wildtype such as Oregon R, with offspring being tested for the presence of the driver line at each generation and subsequently recrossed. Over generations this will lead to high levels of genetic similarity outside of the GAL4 mutation. As such, effects could be more specifically attributed to the differential expression of FOXO rather than any other genetic differences.

However, this was outside the timeframe of this project as it should be performed for at least six generations (Matthew and Partridge, 2016). Taking into account the syncing of eclosure and maturation to the point of sexual maturity, as well as potential developmental variation between lines, a conservative estimate of time to complete the backcrosses would be around three months, assuming there were no issues with the lines.

4.1.7 General comments

Assuming that QMP induces variable insulin signalling levels in the testes, in order for the GAL4/UAS system to result in QMP mediation of sexual fitness the following factors must be true. Firstly, it must be true that QMP impacts insulin signalling in the testes. Secondly, it must be true that *FOXO* levels mediate the impact of nutritional signalling in these tissues. Thirdly, it must be true that the level of *FOXO* expressed in the tissues resulted in *FOXO* levels that sufficiently approximates the physiological range seen in the wildtype insulin signalling system.

In a natural system, with FOXO expression increasing cellular *FOXO* and Akt activity restricting the ability to *FOXO* to enter the nucleus and act as a transcription factor, there is an upper and lower limit naturally found in each tissue based on the level of insulin signalling (Biglou et al., 2021).

In order for the GAL4/UAS system to restore *FOXO* levels and provide insulin sensitivity to a tissue, it must be increased such that it falls within the natural spectrum. If the expression is too low then the repression by Akt will have no meaningful effect due to not being able to noticeably further repress it (Dreyer and Shingleton, 2019 ; Tang, H.Y et al., 2011), and if it is too high it will not be able to repress it to a level lower than the natural maximum level and therefore may not result in a restoring nutritional plasticity. In order for nutritional plasticity to be considered restored, there should be some noticeable difference in function of the tissue between diet-exposed and starved individuals. Furthermore, in order for QMP exposure to be considered to be inducing or not inducing a starvation style effect, there has to be a difference in the diet and starvation-exposed group results in the absence of QMP exposure. This means that in cases where starved and diet-exposed *Drosophila* can not be differentiated, it would not be possible to distinguish between a starvation-like and a diet-like response.

Successfully-mated lines that satisfy the conditions described above would have been required to produce the most comprehensive answers to the research question. However, in practice, some lines failed to meet these criteria in terms of mating success. Nonetheless, in no *Drosophila* line were there instances of statistical significance between

the starvation and QMP group without there also being statistically significant deviation between the QMP group and the diet group. In other words, there was no evidence in any line of QMP inducing a complete starvation-style response distinguishable from diet-exposed flies.

In the lines with direct driver and FOXO parental lineages, it is assumed that insulin sensitivity has been restored to the targeted cell type in the testes and that it can now experience a starvation-like response, including any which may be induced by QMP, if the previous lack of starvation effects had been prevented by low FOXO levels.

In starved flies, by definition, all cells are eliciting a starvation response. In contrast, in the QMP-exposed *Drosophila* there is not necessarily a true starvation response in every cell in the fly. Therefore, if the trigger to perform mating activity originates in a tissue of the *Drosophila* not induced to experience starvation like-signalling or is maintained in a redundant capacity across multiple cell types a tissue, mating activity could still be initiated regardless of the starvation status of the cell type with restored sensitivity to starvation.

4.2 Insulin sensitivity in testes in general

Currently, FOXO mediation of developmental plasticity in male *Drosophila testes* is known to occur in two stages, in genital imaginal disc tissues during larval development (Tang et al., 2011) and the posterior lobe of the genital arch during tissue development (Dreyer and Shingleton, 2019). The restoration of FOXO expression in the testes or testis precursor tissues resulted in less developed testes, which correlate with reduced sexual fitness. This is thought to occur as a result of the tissues developing under a state of perceived nutritional restriction due to the induced nutritionally plastic conditions, resulting in underdeveloped testes relative to those nutritionally insensitive during development.

Whilst the majority of adult tissues develop during metamorphosis and so can be considered to have limited ability to change developmental level following adult eclosure, this is not the case for the sperm cells which are produced during the adult stage of

Drosophila. As a result, it seems possible that different sperm cells have the ability to develop to differing levels of quality throughout the adult stage of *Drosophila*. However, there is likely to be substantial evolutionary pressure to maintain highly competitive sperm due to the high sperm competition found in *Drosophila* matings. This is supported by the low plasticity of the *Drosophila* testes and the existence of substances such as sex peptides which stop females remating with other males following a mating event (Chapman et al., 2003).

Given that the insensitivity of the genital tissue has been found to be due to low susceptibility to insulin signalling, mediated by low *FOXO* levels, it is possible that the same system maintains sperm developmental quality in the adult *Drosophila* males. Given that driver lines for this experiment were selected for their expression across a range of male adult genital cell types involved in sperm production, it is possible to assess if this is likely to be the case in these tissues and to predict in which of the tissues low *FOXO* expression may mediate this process.

4.2.1 Germ line cells (NOS promoter)

The expression FOXO in the germ line cells significantly increased mating success in comparison to the NOSxNOS parental line, indicating substantial impact of FOXO levels on sexual fitness, but not indicating a restoration of plasticity due to mating success levels not differing between diet and starvation groups. The increase in mating success is unexpected given the function of the germ line cells. However, mating success is not necessarily indicative of sperm development, especially if the change in mating success is based on the female assessment of genital development rather than changed mating willingness of the male. It is possible that the increased mating rate is due to the females perceiving the males as more fit. Typically, this is considered to be based on genital development in the male. This theory would make the interpretation of the comparatively low mating success of the parental line difficult as the lack of FOXO expression in the tissue would suggest that the genitals should be fully developed. It is possible that the altered insulin signalling in the germ line cells creates a visible difference in genital structure that is perceived by the female to represent higher sexual fitness and is therefore selected for preferentially, increasing mating success. However, this seems unlikely due to limited visual difference between the tissues, especially when considering

that the NOSxNOS line has substantially lower mating success than other non FOXO expressing lines, pointing to the relative lack of fitness of the NOSxNOS line, potentially due to phenotypic differences in this line not obvious to the human eye that female *Drosophila* view as sexually unattractive.

It is possible that the increase in mating success is due to a last-ditch effort to pass on genetics to the next generation. Given the insensitivity of the testis to insulin signalling, any insulin signalling effect experienced by the cells must be due to substantial levels of nutritional abnormality. This may mean that there is a threshold before absolute starvation that *Drosophila* males are encouraged to mate despite the lack of nutrition to attempt mating before they die as this represents the last chance they have to pass on their genetic material to the next generation. However this theory is unsupported by the literature and it just outlines a theoretical possibility rather than a hypothesis.

Ultimately, the results do suggest that mediating FOXO levels in the germ line cells impacts reproductive fitness, but not that naturally low levels of FOXO in these cells limits their susceptibility to nutrition plasticity.

4.2.2 Somatic support cells (TJ promoter)

The somatic support cells in the *Drosophila* testes surround the developing sperm and provide support, protection (Demarco et al., 2014 ; Zoller and Schulz, 2012) and are potentially differentially responsive to nutrition (Yang and Yamashita, 2015).

The expression of FOXO in the somatic support cells significantly reduced mating success found in both the diet and starvation lines, resulting in mating success results that significantly differ between diets. This is indicative of natural insulin insensitivity in these cell lines mediated by FOXO. No difference was found in post-mating success between diet and starvation in either line, indicating any impact of FOXO expression on sperm quality was not sufficient to impact relative fertility. However, it could still be sufficient in reducing aspects of sexual fitness with regards to function of the sperm such as motility and count which may impact sperm competition ability.

The differing level of mating success following FOXO expression in the somatic support cells suggest that nutritional plasticity in these cells may be mediated by low FOXO levels

and further research in this area may help to improve understanding of the relationship between the genital tissues and insulin signalling.

4.2.3 Late spermatogonia and early spermatocytes (BAM promoter)

When FOXO expression was driven in the developing sperm at the late spermatogonia and early spermatocyte stages, the proportion of successful mating dramatically reduced, but there was no significant difference between diet and starvation exposures. This strongly suggests that maintaining low FOXO levels in developing sperm is highly important in maintaining sexual fitness, despite not inducing a nutritionally plastic response following FOXO expression in these cells.

The results of increasing FOXO expression in the late spermatogonia and early spermatocyte stages suggest that, whilst the level of FOXO is important in maintaining sexual fitness, it is not likely to be protecting these cells from nutritionally sensitive development.

4.2.4 Insulin sensitivity of all three cell types

The reduction of mating success following FOXO expression in these cell types is surprising given the role of the cells in specifying sperm production, as impacting the nutritional sensitivity of the developing sperm would seem more likely to impact the function of the sperm rather than impacting the ability of the male *Drosophila* to mate. The literature typically attributes reduction in mating in less fit males to lower acceptance by females (Dreyer and Shingleton, 2019). However, the effects on fertility of each line was difficult to compare due to limited data points post-mating.

Whilst in all cases there were no obvious differences in the genitalia of the FOXO-expressing lines in comparison to the parental driver lines parental line, it is possible that factors not obvious to the human eye are being valued by the female when identifying the perceived sexual fitness of the male. If the lack of mating is due to a lack of drive to mate this is more difficult to justify. It is possible that the male is able to identify the quality of its own sperm and is less driven to reproduce as a result. However, this is unlikely, there is little evidence for this occurring in the literature and given the natural

insensitivity of the testes to insulin it would be surprising to have behavioural responses in place based on sperm quality, given the inherent resistance of males to reduced sperm quality. Furthermore, whilst less high quality sperm is likely to be less good at producing offspring, it is still the best reproductive option an individual has from a gene conservation perspective to try and mate, given that it is considered the only meaningful way to pass on genes in this species as they are not social.

When taken together, these results point to *FOXO* acting as a substantial specifier in mating behaviour, reducing mating success in the somatic support cells and the late spermatocytes and early spermatoginia cells, and increasing the mating success when expressed in the germ line cells. Restoring FOXO levels even seems to restore nutritional plasticity when expressed in the somatic support cells.

Whilst in the germ line cells and the spermatocytes and early spermatoginia cells, it seems that the lack of difference in the diet and starvation exposures indicates a lack of nutritional sensitivity in these lines. It is possible that this is due to the induced *FOXO* levels falling outside of the range that can be meaningfully modified by the natural insulin signalling system. Quantification of the insulin signalling components in the cell types in the testes would allow for greater understanding of the specific insulin signalling interactions mediating this process. Such approaches include Probe-seq (Amamoto et al., 2019) analysis, which is a bulk RNA sequencing method, if investigating at the cell type level and scRNA-seq (Haque et al., 2017) if investigating at the cellular level. Additionally, improved metrics such as sperm motility and count assays may provide more sensitive assessment of relative sperm quality and be able to predict marginal impacts on sperm performance that may be found as a result of FOXO expression in these tissues. Furthermore, it is possible that the effects are due to drivers expressing FOXO in pre-adult stages which may impact the formation of the genital tissues, resulting in differential fitness. This is unlikely to be happening as the drivers were chosen based upon expression specification in adult tissues related to sperm production. However, expression analysis at each developmental stage with an indicator such as GFP or MCherry would help to confirm this and rule out the possibility of effects being due to expression in pre-adult stages.

Overall, these results act as indicators of potential impacts of FOXO on fitness, suggesting that *FOXO* level is very important in the specification of mating success and that the somatic support cells may be protected from insulin signalling due to low FOXO levels.

4.3 Future Work

Is the effect of QMP due to all of the components; a combination of some components; or an individual component?

Synthetic QMP used in these experiments comprised the main five components of QMP. However, using this blend, it is not possible to distinguish which specific component or combination of components induce QMP responses. In order to investigate this further, subcomponents of QMP could be administered individually and in different groups in order to understand if any induced responses are the result of an individual chemical or a group synergistic effect. This is especially relevant in the assessment of QMP on male reproductive success as 9-ODA, one of the components of QMP, is specifically attributed to the attraction of drones to queen bees and so may independently impact male *Drosophila* sexual fitness in isolation from the rest of the components of QMP (Gary, 1962; Gary and Marston, 1971; Brandstaetter et al., 2014). Equally, there may be levels of redundancy within the QMP components, which would be indicative of the complexity of the induced signalling.

Cell type-specific quantification of insulin signalling following QMP exposure

As mentioned in the discussion, probe-seq (Amamoto et al., 2019) and scRNA-seq (Haque et al., 2017) techniques would allow quantification of components of insulin signalling in specific cell types to be known. This would allow quantification of the cellular signalling impacts of QMP exposure on male *Drosophila,* giving greater insights into the cell type-specific responses found in each cell and the level of mediation attributable to low *FOXO* in each cell type. When compared with the results of the mating assay, this would allow greater understanding of how the response acts through these tissues and to what extent each cell type may be involved.

Investigating the effect of QMP exposure on drone fertility

Whilst *D. melanogaster* research facilitates comprehensive genetic work and ease of experimental procedure, ultimately they are used in QMP research to facilitate understanding of how QMP works in honeybees. As such, the greater the understanding of the impact of QMP exposure on drone fitness, the greater the understanding of the impact of QMP on male *Drosophila* can be contextualised. Whilst *Apis. mellifera* drones are not considered to be reproductively repressed by QMP as they are exposed to it throughout development and are still able to successfully fertilise the queen, this supposition has arguably served as a barrier to research, and the absolute effect of QMP on the sexual fitness of drones remains unknown.

In order to further understand the impact that QMP has on drone fertility, drones could be reared in natural honeybee colonies / exposed to synthetic QMP / not exposed to QMP. Following rearing, metrics of sexual fitness could be assessed, including sperm count and motility analysis. Furthermore artificial insemination of queens using sperm from reared drones would allow quantification of the in-vivo fertilisation rate. This would need to be conducted using pooled sperm samples as only about 1μL of sperm can be collected per drone, with 8-12 needed per insemination (Khan et al., 2022). Following insemination, fitness metrics such as offspring production and success of adult offspring development could be measured. When considered together, the combined sexual fitness metrics would facilitate assessment of the relative fitness of each exposure type.

Section 5 - Conclusion

QMP induces a starvation effect in male *Drosophila*, similar to that seen in females (Lovegrove et al., 2023). However, unlike in females, it is induced during, not following exposure. This induction of feeding may explain the high phenotypic frequency of abdomens thought to correspond to full crops. This phenotype is very similar to that seen in starved flies that have been recently exposed to food (Wong et al., 2008) and so may indicate that QMP is inducing a starvation-style effect that, due to the presence of food, results in enlarged crops. This might explain the lack of feeding post exposure as these full crops might reduce further feeding ability, or that any induced starvation does not persist outside of direct QMP exposure.

Activity is also impacted by QMP exposure, with a reduction found in QMP-exposed males relative to non-exposed and starved flies across both day and night. This may be partially due to some of the factors that cause the increased mortality seen in QMP-exposed male *Drosophila* over a 48h period compared to non exposed groups, although this impact can not directly explain the reduction in activity due to no deaths observed over the 24h activity monitoring period in the activity assays. There is some evidence that if QMP is impacting insulin signalling, as is seen in females (Lovegrove et al., 2023), life expectancy could be impacted (Clancy et al., 2001). However, the increase in mortality at 48 hours seems to be more indicative of a toxic effect rather than accelerated ageing.

When comparing the relative sexual fitness of QMP-exposed *Drosophila* males with restored insulin sensitivity in the sperm producing cells, in the FOXO expressing lines, QMP affected mating success in TJxFOXO in which FOXO was expressed in the somatic support cells and the chance of offspring production in *Drosophila* with *FOXO* in the germ line cells under the expression under the control of NOS. In non FOXO driven lines QMP was only found to impact sexual fitness metrics in one driver control line (TJxTJ), in this line mating success was reduced relative to both controls. Mating latency and chance of offspring production were also impacted by QMP exposure in the UAS-FOXO parental line.

The impact of QMP on sexual fitness is complex and induced responses differ by cross. However, despite the differences found in the effect of QMP exposure in the FOXO expressing groups compared to that found in the parental control lines, no groups of either type showed starvation-like responses, indicating that the induction of a starvation effect QMP in these cell types is unlikely and that low levels of FOXO in these cell types is unlikely to be the reason that QMP can not induce reproductive repression through these tissues. However, due to low mating rates in the assay and difficulties with the stocks of the NOSxNOS parental line, post-mating sexual fitness metrics in some instances are not possible to analyse.

Finally there is some evidence that in wildtype *Drosophila,* nutritional sensitivity of the developing sperm is mediated by the insensitivity of the somatic support cells to insulin sensitivity due to low levels of *FOXO*.

Further research into the levels of insulin signalling in each cell type through processes such as probe-seq (Amamoto et al., 2019) and scRNA-seq (Haque et al., 2017) analysis could allow for better understanding as to how FOXO levels react to changes in insulin signalling in each cell type if used to compare diet and starved groups, which would allow for identification of cells naturally resistant to insulin signalling that may mediate the difference in QMP effect on sexual fitness by sex. Furthermore, by performing this experiment with a QMP exposed group it could be assessed if QMP induces insulin signalling at all in the genitals of male *Drosophila* which is still currently unknown.

This research contributes to the greater understanding of pheromones of and therefore communication in social insects and may help in the comprehension as to the factors that facilitate the existence of social species, which display the most complex form of social interaction across the animal kingdom.

Section 6 - Appendix

6.1 Accompanying p values for graph plotted 3.1.

Point analysis graph of point absorbance post exposure feeding results.

	Exposure.diff	Exposure.lwr	Exposure.upr	Exposure.p.adj
ETHS-ETHD	0.7440462429	0.5866867	0.9014058	2.01E-09
QMPD-ETHD	-0.0004624278	-0.157822	0.1568971	1.00E+00
QMPS-ETHD	0.6182658957	0.4609064	0.7756254	2.94E-08
QMPD-ETHS	-0.7445086706	-0.9018682	-0.5871491	1.99E-09
QMPS-ETHS	-0.1257803471	-0.2831399	0.0315792	1.43E-01
QMPS-QMPD	0.6187283235	0.4613688	0.7760879	2.91E-08

6.2 TGPH4 visualisation protocol

In an effort to categorise the effects of QMP exposure as either starvation-like or not starvation-like a TGPH4 mutagen line was used. Male flies were collected as virgins and aged for 24 h before 48 h exposure to diet/starvation. Following exposure, flies were anaesthetised over ice and dissected in 1xPBS solution on ice removing the testes (Figure 32).

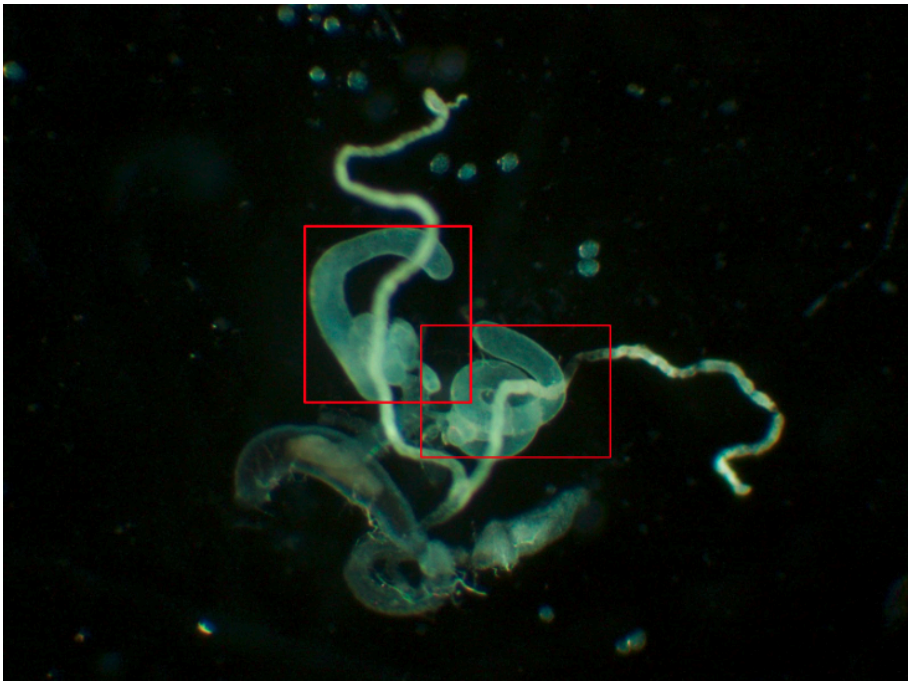


Figure 32. **Diagram of testes of Drosophila melanogaster.** Testees extracted from TGPH4 mutant *Drosophila melanogaster* using a dissection microscope in 1x PBS, visualised using an attached GXCAM imaging camera with 1.7x objective and 10x eyepiece magnification.

Testes were stored on ice in 400µL 1xPBS until all dissections were completed. Following this, 350µL of PBS was removed and 900µL 1xPBS and 4% Formaldehyde solution were added to the microcentrifuge tube which was then rocked for 10 minutes at room temperature. As much of the solution as possible was then removed before four washes with PTx (Phosphate Buffer Saline with 0.1% TritionX 100). The solution was then removed and 1mL of PTx and 1µL DAPI (a fluorescent stain which binds to DNA, staining the nucleus of cells) was added and mixed using inversion. This was then stored in darkness for 10 minutes. Following this, two washes were performed with PTx. Samples were then isolated from the solution using a pipette and stored in 70% ultrapure glycerol at 4°C overnight before visualisation.

Immediately prior to visualisation, testes were removed from the solution placed on a slide with a coverslip placed on top. Clear nail varnish was used to seal the edges of the coverslip. Prepared slides were shielded from light outside of visualisation. An EVOS Auto2 microscope was used for visualisation, recording levels of absorbance of DAPI and GFP at magnifications of 2x, 4x, 10x, 20x and 40x. This was controlled using the FL Auto 2 software.

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