The Pheromonal Basis of Reproductive Constraint in Eusocial Insects

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I confirm that the work submitted is my own, and that appropriate credit has been given where reference has been made to the work of others.

For the bees,
without whom, all of this is meaningless

Acknowledgements

In order to properly thank all of the very many people without whom I could not have completed this thesis, there is an implicit understanding of the “hierarchy of thanks” that comes with ordering and length of the people about to be mentioned. Ignore this. There is no hierarchy. While I have worked with some more and some less, and have been supported more and less by different people over the last four and a half years, every single person forms a part of my identity here in Leeds, and I will carry those relationships within me wherever I go.

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Abstract

The reproductive division of labour is a key characteristic of eusociality. In *Apis mellifera* honeybees, this is maintained in large part through the secretion of pheromones, particularly by the queen, which prevent the sexual development of workers. The aim of this thesis was primarily to investigate the mechanism of action of the principal reproductively constraining honeybee queen pheromone: Queen Mandibular Pheromone (QMP). The secondary aim was to investigate the role of less well-studied pheromones, such as brood pheromone (BP) and E-β-ocimene (EBO), on reproductive constraint.

Although many disparate elements of QMP-mediated physiological effects have been characterised, there has yet to be identified any unifying mechanism which explains how the physical interaction of a worker honeybee with QMP is able to bring about reproductive repression of worker ovaries. Similarly, QMP can act incredibly widely across the insect orders, repressing reproduction in the bumblebee *Bombus terrestris* and the fruit fly *Drosophila melanogaster*, and it is unknown whether QMP is acting via the same mechanisms in these other insect species as it is able to act in honeybees. Additionally, although the larval pheromones BP and EBO have been shown to bring about reproductive repression in workers, the mechanism by which these do so is completely unknown.

In chapter 3 I investigate different elements of the effect of QMP on honeybees at a physiological level, demonstrating that honeybees lack plasticity in their adult reproductive constraint mediated by QMP, and that QMP likely induces starvation in honeybee workers in a similar manner to fruit flies. In chapter 4 I attempt to establish an *in cavea B. terrestris* model for investigating the mechanism of action of QMP and pentacosane in this species. In chapter 5 I demonstrate a lack of reproducibility for the published work showing that BP and EBO bring about reproductive repression in honeybees, and show that these pheromones do not induce reproductive constraint in *D. melanogaster*. In chapter 6 I investigate the plasticity of QMP effect in *D. melanogaster*, and identify that diapause is likely the mechanism through which QMP is able to bring about repression of reproduction in this species, but that the repression is likely mediated through different tissues than cold-induced diapause.

Contents

List of Figures

List of Tables

List of Abbreviations

QMP – Queen Mandibular Pheromone

BP – Brood Pheromone

9-ODA - (E)-9-oxodec-2-enoic acid

HAD - (E)-9-hydroxydec-2-enoic acid (R/S)

HOB - methyl p-hydroxybenzoate

HVA - 4-hydroxy-3-methoxyphenylethanol

EL - ethyl linoleate

ELN - ethyl linolenate

EO - ethyl oleate

ES - ethyl stearate

EP - ethyl palmitate

ML - methyl linoleate

MLN - methyl linolenate

MO - methyl oleate

MS - methyl stearate

MP - methyl palmitate

EBO – E-β-ocimene

PBS – Phosphate Buffer Saline

n-C25 – Pentacosane

CBF – Complete Bee Food

QC – Quality Control

Qe – Queen equivalent of pheromone

FandP – Fondant and Pollen Diet

RT-qPCR – Reverse Transcription quantitative polymerase chain reaction

Chapter 1 Introduction

1.1 Insects and Insect Phylogeny

The diversity of form and function in the insects truly embodies the concept of Darwin's "endless forms most beautiful and most wonderful"(1). They are the most abundant class, and with greatest species richness across the metazoans (2).

Emerging during the Ordovician, at the same time as land-dwelling plants (~450-470 mya) (3), the insects went through several major radiation events. The first flighted insects emerged in the Devonian period (~400 mya) (3).

The Pterygota (originally winged-insects, though many lost their wings later) underwent a major radiation event in the Carboniferous (~300-350 mya) (4), and a subsequent radiation event of the holometabolous insects (the Endopterygota, those insects which undergo complete metamorphosis (5)) occurred during the Permian expansion event (~250-300 mya)(3). After the mass-extinction event of the Permo-Triassic boundary (6), the insect orders have remained largely stable (3).

Insect families begin to stabilise during the Jurassic period (~150-200 mya) (3) alongside the evolution of the flowering plants in the Cretaceous period (~65-150 mya), resulting in the coevolution of some of the more characteristic insect families, genera, and species seen today, such as the Coleoptera (beetles), Lepidoptera (butterflies), some Diptera (true flies), and the Hymenoptera (bees, ants, wasps, and sawflies) (3, 7).

A phylogeny of the insects is reproduced from Misof et al. (3), in Figure 1.1, depicting the evolutionary relationships of the insects.

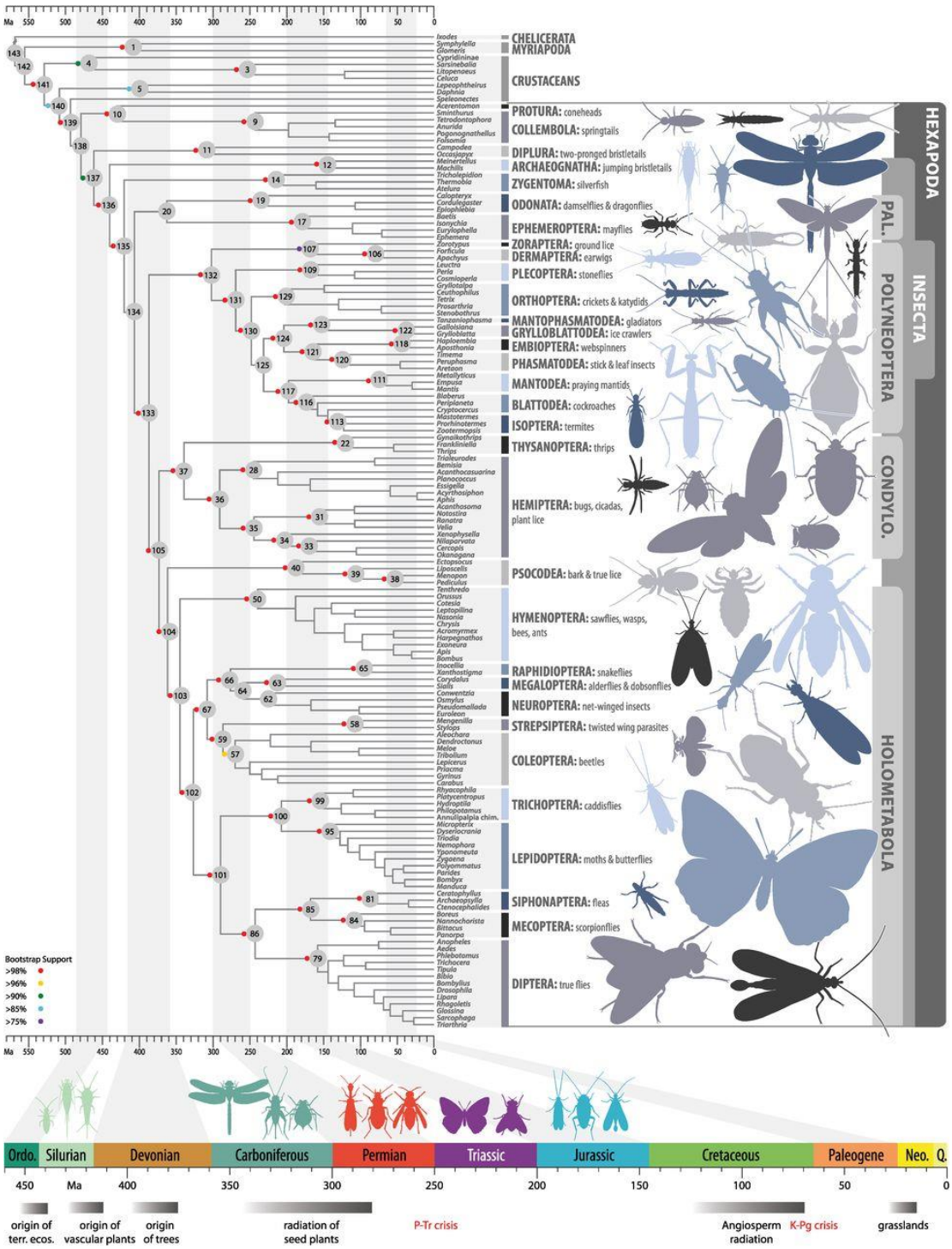


Figure 1.1 – Showing the phylogeny of the insects Reproduced from Misof et al. (3), the phylogenetic tree shows the phylogeny of all extant insect orders, along with the geological periods and broad biological trends of the given time period.

1.1.1 Phylogeny of the Hymenoptera

As mentioned previously, generally the main orders of modern insects first appear in the Permian, which is also true for the common ancestor of the Hymenoptera (7). Within this order, the evolution of parasitoidism during the Permian-Triassic extinction event marks the start of the evolution of the parasitoid wasps, with the stinger evolving from the ovipositor at some point in the late Triassic/early Jurassic. At this point the lineage splits into the ancestors of the social wasps, and the ancestors of the ants and bees, which occurs in the early Jurassic. The first evolution of eusociality in this order occurs in the Cretaceous (~140 mya), resulting in the evolution of ants.

Within the Apoidea (the superfamily containing the sphecoid wasps and the bees) the sphecoid wasps (e.g. mud daubers) and the bees diverged in the mid Cretaceous (~125 mya). The seven extant families within the Anthophila (the bees) continue to stabilise until the evolution of eusociality in this family in the early Paleogene period (~65 mya) (8). Within the Apidae family, the Apis (honeybees), and Bombus (bumblebees) genera diverged towards the end of the Paleogene (~35 mya).

An overview of the phylogeny of the Hymenoptera is given in Figure 1.2.

Among the many characterising features of this order, such as the evolution of a narrow waist and stingers, is the evolution of eusociality. Indeed, there are several points during the evolution of this order where eusociality begins to appear. Eusociality has evolved many times within the Hymenoptera, with estimates changing due to consistently updating phylogenetic analyses of the order. (7, 9-18)

Within the *Apidae* family there is thought to be only a single origin of eusociality (18).

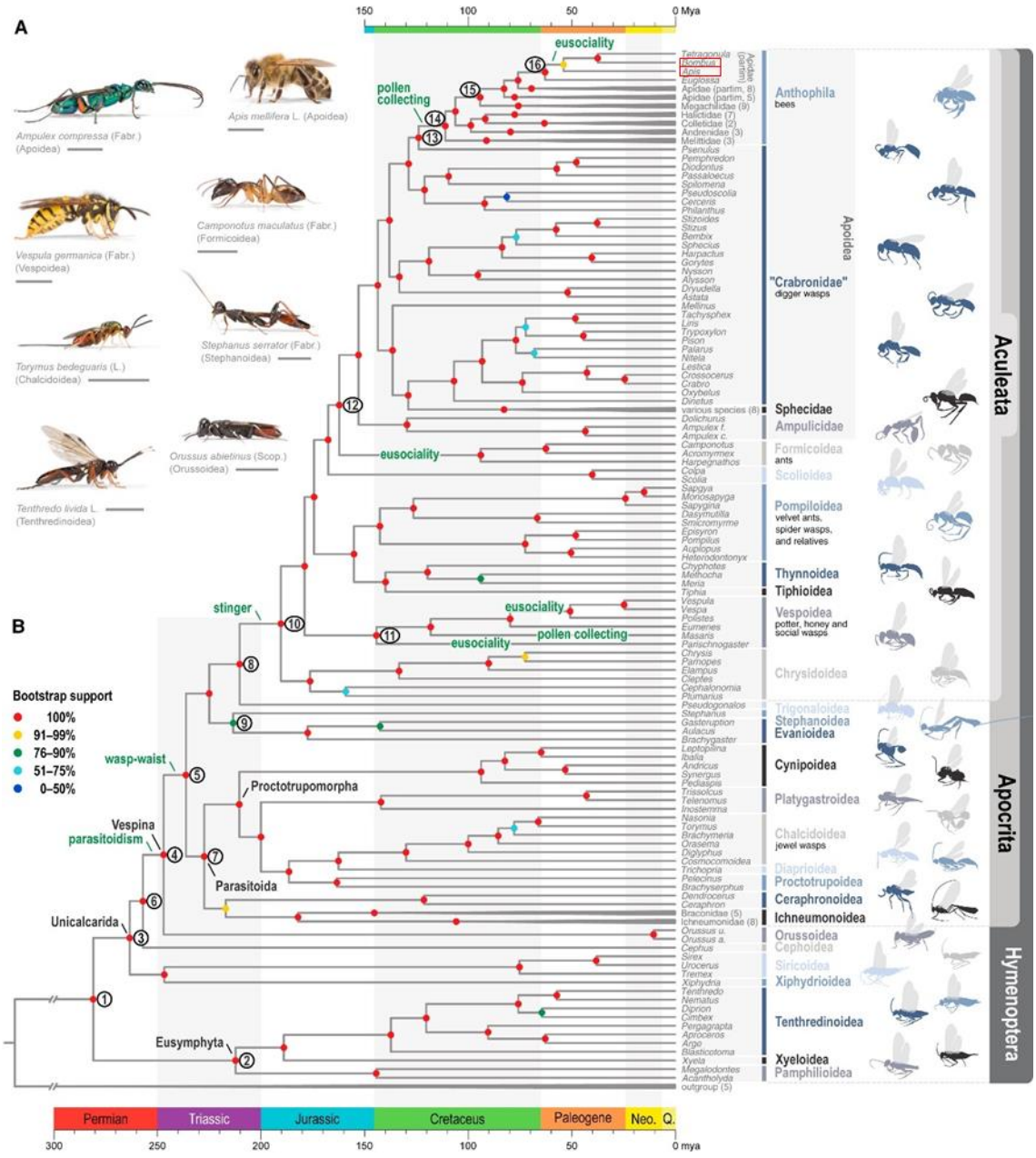


Figure 1.2 – Showing the phylogeny of the Hymenoptera Reproduced from Peters et al. (7), the phylogenetic tree shows the Hymenoptera, as well as the predicted key evolutionary events marked in green. A geological timeline is given at the bottom, as well as example Hymenopteran species in the top left. The two genera used in this thesis are highlighted in a red box.

1.2 Eusociality

Eusociality is one of the eight major transitions in evolution characterised by John Maynard Smith (19). It is a form of complex social organisation originally characterised by three criteria, but principally by the reproductive division of labour, in which a subset of a social population takes on the responsibility of reproduction and is aided in that by non-reproductive individuals. These systems also possess co-operative brood care, as well as overlapping generations (20). Other characteristics have also been associated with eusociality in insects more specifically, such as trophallaxis of fluids (21), but are not widely accepted as the minimum definitions needed to define this social system.

Another common characteristic of more complex eusocial systems is the evolution of morphologically distinct castes. The simplest of these is the distinction between the reproductive queen, and the non-reproductive worker, which can be based in size, physiology, and anatomy, for example the queen of a honeybee colony is larger, possesses a different set of reproductive anatomy, as well a differently shaped sting (among many other notable features), when compared to that of the workers (22). There can also be other caste variations in the workers, such as in many species of ant, which can possess a “soldier” caste, characterised by morphology specialised for colony defence (23), and subcaste variations, such as major/minor workers in species of ant such as *Messor barbarus* (24) though these classifications are disputed.

There is growing criticism of the definitions used herein (e.g. (25)), mainly directed at the nebulosity of terms such as eusocial, superorganism, and complexity. These terms are often used with limited consistency, with some researchers advocating that they should be rejected for more consistent, objective language; however, the language changes proffered bare the same issues as the words they are attempting to replace (such as the use of the word “social” to replace “eusocial” (26)). Although there is dispute over the terminology, the most widely accepted view is in acceptance of these terms, and so they will be used throughout due to the importance of separating the types of social organisation seen in eusocial systems and the types of organisation seen in simpler social systems (such as colony forming seabirds, like the puffin (27)).

1.2.1 Characterisation of Eusociality

Different eusocial systems can be characterised in ways which allow for a spectrum of complexity to be categorised (20) (Figure 1.3). From cooperation between individuals in facultative social organisation at one end (28), to highly derived superorganismal eusociality at

the other (29). The middle steps of this ladder are filled with examples of individuals which have, for example, only partial reproductive division of labour, or conditional cooperative brood care; which are often called primitively eusocial. (30)

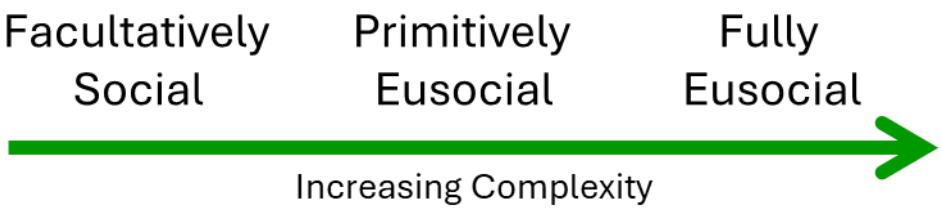


Figure 1.3 – Showing a simplified diagram of the spectrum of Eusociality

1.2.1.1 Facultative Sociality

Facultatively social species have many of the basal social characteristics of eusocial systems, but have not evolved the reproductive division of labour. An example of such a characteristic is the social aggregation of nest sites together, as in the social spider *Malos gregaris* (31), but this may also manifest in solitary lifecycles with dispersion, as in many solitary bees, where the opportunity to remain *in situ* occasionally occurs, as in the facultatively solitary bee *Ceratina australensis* (32). In this species, the offspring of the female generally disperse to colonise more nesting sites in a solitary manner, with a reproducing female only ever occupying a single nesting site, occasionally, at a rate of about 13% of nests, one daughter will remain and provision the same nest site as her mother (33), though the maximum number of individuals provisioning a single nest is two (34).

In facultatively social species, there is no caste separation (i.e. there are no specific tasks carried out by specific individuals, and no reproductive division of labour), though there may be dominance hierarchies which do not lead to reproductive division of labour. Morphologically the individuals are indistinguishable.(35)

1.2.1.2 Primitive Eusociality

Some species display greater degrees of social organisation, and display some degree of caste separation in the reproductive division of labour (36), or engage in full reproductive division of labour some of the time (37, 38). In this social grouping, there may be some morphological difference between castes, particularly between the queen, and workers.

This grouping has perhaps the greatest variety of different species and life strategies, and is the least useful descriptor for social species, as it broadly covers every form of species which exhibits greater eusocial tendency than a facultatively social species, but is not fully eusocial.

The primitively eusocial paper wasp *Polistes dominula*, share no morphological differences between queens and workers. Multiple queens often found colonies, though with dominant and submissive queens according to reproductive output. The submissive queens also do worker tasks, with the offspring of these queens being workers and not reproducing. Initially the colony founding includes many tasks conducted by the founding queen. (39)

A second primitively eusocial species is the *B. terrestris* bumblebee. This species has considerable morphological difference between queen and worker, as well as polymorphism-determined ethology in workers (with larger workers foraging, and smaller workers performing internal colony tasks). Reproductive division of labour is present for much of the colony lifecycle, however during the final third of the lifespan of this species, reproductive division ends and workers are able to lay eggs which produce males (these male destined eggs are due to haplodiploid sex selection in this species, discussed in section 1.2.2.2).(40)

1.2.1.3 Complete Eusociality

In the complete eusocial species we see all the three defining aspects of a eusocial species: Reproductive division of labour, cooperative brood care, and overlapping generations (20). There is often complete morphological separation of the worker and queen castes, often with workers lacking the ability to reproduce entirely, as in the ant species *Solenopsis invicta* (41).

One of the most well studied eusocial species is the Western honeybee *A. mellifera*. In this species workers are still able to reproduce, and under certain circumstances will lay eggs, but usually only if the queen has died and attempts to replace her have failed (42). Though there is no polymorphism amongst workers (i.e. no significant, or role-related, morphological variation in workers), there is relatively strict temporal division of labour (with younger bees performing internal colony tasks such as brood care and cleaning, and as they age they switch to external colony tasks like hive defence and eventually foraging) (43), though the bees in a colony will adapt to their colony needs (44). In this species queens are fully separated in social role, and never engage in colony management, nursing, or foraging tasks, strictly only laying eggs (this includes never stinging, despite possessing the ability to) (22).

The honeybee colony does not reproduce through gyne production and dispersion, as with many primitively eusocial species, but through splitting, whereby a new queen is produced and the older bees and queen remove themselves to a new hive location, leaving behind the new queen and younger bees (45). This process is known as swarming.

Fully eusocial species also, by necessity, tend to have a much more rigid set of mechanisms through which the prevention of reproduction of workers occurs (46). For example, the

morphological lack of reproductive organs in workers, or the presence of potent reproductive inhibitors such as queen pheromones (47). This form of social organisation is also associated with much larger colony sizes, potentially up to trillions in the case of the Argentine ant *Linepithema humile (48)*, though more normally in the tens to hundreds of thousands (49).

1.2.1.4 The Superorganism

An element of eusociality which both allows us to model the nature of a eusocial insect colony, and to better contextualise the difference between primitive eusocial species and merely facultatively social species, is the concept of the superorganism (20, 50).

In this scheme, the individuals which comprise a colony can instead be considered the constituent parts of the whole. In that manner the colony can be modelled as the individual, a quasi-multicellular organism in which the cells are the constituent workers and queens (with the workers being the somatic cells, and the queens the germline cells)

In the structure of the superorganism, the nature of its evolution occurs not at the individual level, but at the group level, selecting for traits and phenotypes not linked to the genotype of the workers but of the colony as a whole (30). The fact that the workers are all the offspring of the queen allows for the colony-level selection of traits which maximise cooperation between sisters. It is from this cooperation that the unique ethology of eusocial insects develops, with the true reproductive division of labour that occurs (51). To the worker and the queen both, the reproduction of the colony is equivalent to their own reproduction (20). The “group-selection” element of these evolutionary selection pressures does not necessarily contradict the fact that these pressures are applied to individuals, but rather that the nature of the complex social interactions that exist between individuals produce additional opportunities for selection pressures to be applied (for example the constant temperature homeostasis that exists within honeybee colonies (52) is a form of superorganismal trait on which selection will act). It is often more useful to model these traits from a group-selection perspective, as that is the level at which the phenotype is observable.

Although there are many possible analogies to be drawn from other contexts for explaining this, such as in humans: the gametes of our reproductive organs are the queens, while the rest of our cells are the workers facilitating this; there is a danger in over-relying on these analogies. In a eusocial colony individual workers retain a fair degree of autonomy and in some species can for example reproduce under queenless conditions (53, 54). A eusocial species has the potential to revert to primitively eusocial, or lose its eusociality altogether, which has happened in the ants (55), though this process is particularly rare.

Similarly, workers of some species often have the capability to migrate to colonies to which they are unrelated (such as in honeybees)(56), and in some genera workers can be freely swapped between species with no ill effect, such as in the *Formica* genus of ants. Indeed, some ant species, such as the slaver ant *Formica sanguinea* have taken to this as a life-strategy and engage in slave raids on other ant species in their environment (57). This horizontal transfer of individuals is only possible between closely related groups (as in the *Formica* which are all diverged within the last 25 million years (58)), underscoring the difficulty in using allegory to understand this sociobiological phenomenon, as this simply does not occur in any analogous context.

It is also possible to draw analogies to human societies in this manner, considering human cultures and tribes, or racial groups, to be equivalent to the superorganismal nature of eusocial insect colonies, however this falls trap to the anthropocentric fallacy (59), and we should assess this form of social organisation based on its own merits and characteristics, rather than clouding our understanding by analogy and allegory.

1.2.2 Evolution of Eusociality

Although the successfulness of eusocial systems is eminently evident in nature, and have they evolved many times independently, eusociality as a social system is not ubiquitous across the tree of life. There are notably very few, or no depending on strictness of definitions used, eusocial societies in e.g. mammals, reptiles, or fish.

Understanding why eusociality is as relatively abundant in the insects as it is (though noticeably still a minority of species), particularly in the Hymenoptera, is therefore an academic question of particular interest. Indeed, Darwin himself called this social system the “one special difficulty” with his theory of evolution by natural selection (1), due to its seeming contradiction with the concept that each individual acts in its own, selfish reproductive fitness interest.

1.2.2.1 Kin-selection

Hamilton formalised the way in which eusocial systems can evolve at a theoretical level, with his defining of what is now known as Hamilton’s rule, which conceptualises how altruism can exist within Darwin’s theories revolving an organism always acting in its own interest. This states that animals who are related to one another, gain indirect reproductive fitness via the offspring of the relative. In this way, one can balance an act deleterious to the individual’s direct fitness with the indirect fitness gained by this act: $RB > C$. (Where the relatedness R, multiplied by the benefit B, must be greater than the cost C).(60)

Although this provides the framework within evolutionary theory to allow for the evolution of eusociality, it does not provide a mechanism nor explain the higher rate of insect eusociality versus other orders of animals.

1.2.2.2 Haplodiploid Sex Selection

In the Hymenoptera, sex selection plays a mechanistic role in kin-selection, via haplodiploidy.

A genetic quirk of the Hymenoptera is that sex is determined by ploidy: specifically, that generally males are haploid and females diploid. Within monandrous pairings, this gives rise to a relatedness asymmetry as shown in Figure 1.4. On average, females in haplodiploid species inherit 50% of their genome from their mother and 50% from their father – which is 100% of their father's genetic information, as a result they all share at least 50% of their genetic information with one another, and up to 100%, if they inherited the same alleles from their mother; though this averages to 75% across a population. This makes mothers and daughters only 50% related, but sisters 75% related. Meaning that workers are able, on average, to greatly increase their own reproductive fitness by supporting the reproduction of the queen. They are also better off supporting the reproduction of their sisters over their own children. (61)

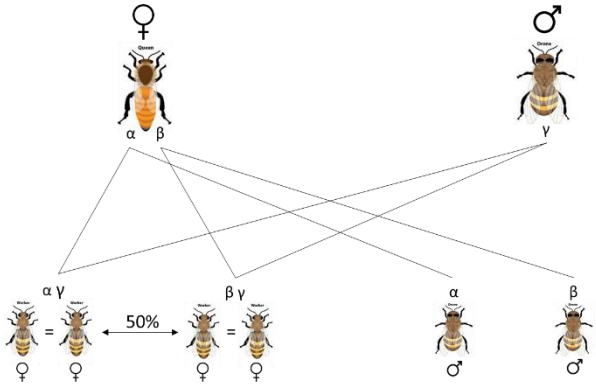


Figure 1.4 – Showing a diagrammatic explanation of how haplodiploid sex-selection results in greater relatedness between sisters than between offspring and parents.

With polyandry, whereby the queen mates with multiple males, relatedness between sisters instead tends towards 50%, undermining haplodiploid-mediated kin-selection. However, it has also been postulated that monandry is the ancestral state of all eusocial hymenopterans (10), and that polyandry could only have occurred after eusocial systems had evolved such that they are impossible, or very difficult to revert to facultatively social organisation. The evolution of polyandry is a mechanism through which increased genetic diversity can come about. This is

important, due to the reduction in overall genetic diversity that occurs as a result of eusocial evolution. (10)

1.2.2.3 Pre-adaptation for eusocial evolution

A holistic model understanding how eusociality was able to evolve is the pre-adaptation theory. Species with similar selection pressures will develop similar traits to maximise their fitness, and in some, combinations of these traits lays the foundational groundwork, which, when the correct selection pressure is applied, can result in the evolution of a eusocial system. (30)

These traits include the formation of groups (e.g. the defensible nests of the eusocial bamboo aphid (62)); willingness to divide labour, when introduced into a social setting (as in some solitary *Lasioglossum* bees (63)); nest provisioning, whereby an individual stores provisions for other individuals (as in many parasitic wasps (64); and parental care of brood (as in burying beetles (65)).

This theory provides opportunity to contextualise the “missing links” between facultatively social and eusocial evolutionary species.

It is perhaps worth observing that in all of the models and explanations provided hitherto, general concepts are trying to be outlined. In actuality, eusociality is a phenotypic observation, and all of the independent origins of eusociality will have evolved through different evolutionary paths by virtue of having occurred in different times and places. The pre-adaptations that are proposed for the evolution of eusociality are differentially important relative to the ecological niche of the species, the selection pressures that are applied on them, and the social challenges that were met with increased social cooperation, at the expense of genetic diversity and individual fitness. It is possible that there are extreme degrees of overlap between these independent origins, both within the Hymenoptera and outside of it.

It is certainly true that the traditional explanation, that eusociality is principally caused by kin-selection, has been shown to be a small part of a much larger picture (30).

1.2.2.3.1 Reproductive Ground Plan Hypothesis

An example of a pre-adaptation that seems to be necessary in the hymenopteran insects for the evolution of eusociality was proposed in the reproductive ground plan hypothesis (66, 67). In this theory, the gene networks that regulate foraging and reproduction in solitary insects are coopted in the evolution of eusociality.

In particular, gene networks involved in the maintenance of reproduction in the solitary ancestor, such as vitellogenesis during laying periods, have been coopted into allomaternal

behaviours in honeybee workers (such as brood rearing). These reproduction-linked networks in solitary ancestors have been decoupled from reproduction in the worker, but retain links to reproduction-adjacent tasks, such as food provisioning, and other allomaternal behaviours (68-70). Interestingly, this decoupling does not occur in queens, which retain the effects of these networks as in the ancestral examples (71).

For example, in solitary insects there are general periods of reproductive activity, requiring higher vitellogenesis (72), and as a result the endocrine and gene network states that allow this (such as low juvenile hormone titres (73)). Antagonistic cycles of high juvenile hormone and low vitellogenin (which occurs during foraging), and low juvenile hormone and high vitellogenin (which occurs during reproductive periods) drive the necessary physiological changes which governs this reproductive and foraging behaviour (67). The same hormone and gene network relationships can be observed in the honeybee (74, 75), however they are tied not to reproduction and foraging, but internal and external hive roles (nursing and foraging respectively) (76). In queens however, the antagonistic relationship between juvenile hormone and vitellogenin with regards to reproduction remains the same as in the ancestral state, whereby high juvenile hormone and low vitellogenin is associated with non-reproducing individuals (such as virgin queens, or those queens prevented from laying eggs, e.g. by caging them), and low juvenile hormone and high vitellogenin with reproductive individuals (such as mated and laying queens) (71).

This decoupling of these hormonal and genetic responses to reproduction in honeybee workers, but not queens, represents a mechanism which may have been co-opted, or possibly driven by, the evolution of queen pheromones in order to induce reproductive constraint.

1.3 Pheromones

Pheromones are a form of chemical communication used across the entirety of the animal kingdom (77). They can be used as: trail agents, to lead nestmates to food, as in ants (78); as territorial markers, as in domestic cats (79); as sex pheromones to attract a mate, as in bees (80); as aggregation markers, as in bed bugs(81); as alarm chemicals, to alert others to danger, as in sea anemones (82); even Nasonov pheromone, which is used by honeybees as a beacon to attract swarms to potential hive locations, and to attract foragers back to the hive (83).

Pheromones can be divided into two major groups relating to their function, releaser pheromones and primer pheromones.

Releaser pheromones are those that cause an immediate behavioural response in the target organism e.g. the aforementioned trail pheromones; whereas primer pheromones are those

that cause an innate physiological change that can result in a behavioural change, but not necessarily immediately e.g. queen pheromones, which can be used to supress the reproduction of workers in eusocial insects by suppressing ovary development. (77, 84)

Pheromones are the most important form of communication in eusocial insects, with a hugely diverse range of functions (85). Their role in alarm, trail laying, sex attraction, nestmate recognition, brood care, aggregation, and swarm guidance, are subject to continual research.

1.3.1 Queen pheromones

Of all the pheromones produced by hymenopteran insects, the queen pheromones have been studied most for their role in the maintenance of eusociality via the repression of the reproduction of workers.

In most Hymenoptera, these pheromones are cuticular hydrocarbons (CHCs), a set of long chain and sometimes simply-branched hydrocarbons, whose ancestral role is thought to be in preventing desiccation (86), and are also important in nest mate recognition (87). There is some small overlap between species as to which CHC acts to suppress ovary development, though bumblebees have noticeably different queen pheromones to other types of eusocial insects, e.g. heptacosane (nC-27) produces significant ovary repression in both the yellowjacket wasp *V. vulgaris* and the desert ant *C. iberica*, which have independent origins of eusociality (7), but does not produce significant ovary suppression in *B. terrestris* bumblebees (88).

That CHCs neither display rigorous specificity nor broad phylogenetic effect with regards to their reproductive repression is likely related to the evolution of their repressive role. It is thought that CHC profiles are associated with certain reproductive states, with greater fertility of the queen being shown in the nature of her CHC profile, and so therefore bring about reproductive constraint only within the context of them being honest signals of fertility (46, 89)(discussed later in section 1.4 in more detail), and so do not diverge very quickly from their ancestral form. Thus, even species which are not closely related, like *V. vulgaris* and *C. iberica*, are able to share common ancestral signals of fertility and their effect, but the more distantly related *B. terrestris* is unable to be affected in this way (46). Notably the bumblebee *B. impatiens* was shown to be responsive to the CHC profile of its own queen, though only when combined with the physical presence of the queen (by sight), indicating that context is important for this species (90). This might explain the lack of effect seen in *B. terrestris*, however this has not been tested.

Unusually, the honeybee genus *Apis* has much a much more complex queen pheromone, when compared to the CHC queen pheromones of the other eusocial hymenopterans. While the

CHCs are simple, often unbranched long chain hydrocarbons with very few functional groups, QMP possesses many structurally complex hydrocarbons with several functional groups and aromatic compounds. The similarities and differences can be seen in Figure 1.5, where the CHCs are shown in red, and the QMP components in green, and where the difference in complexity between these two classes of compounds is clear.

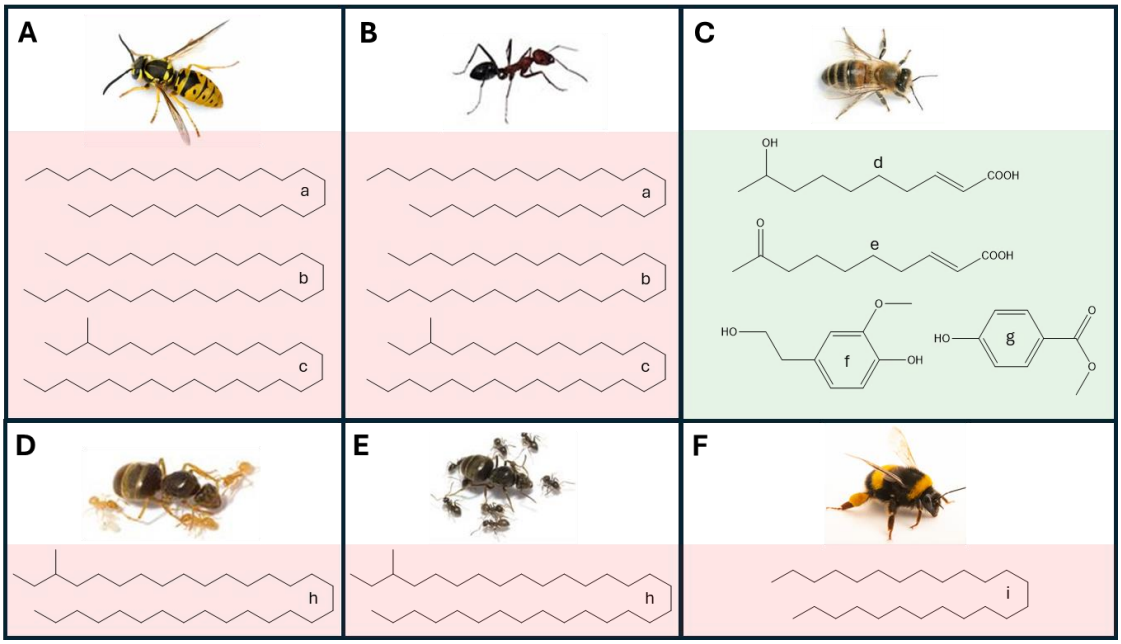


Figure 1.5 – Showing the different queen pheromones of different species of Hymenoptera Each box shows a different species: **A**: *Vespula vulgaris*; **B**: *Cataglyphis iberica*; **C**: *Apis mellifera*; **D**: *Lasius flavus*; **E**: *Lasius niger*: **F**: *Bombus terrestris*. Each pheromonal compound is also labelled: **a**: heptacosane, **b**: nonacosane, **c**: 3-methylnonacosane, **d**: 9-HDA, **e**: 9-ODA, **f**: HVA, **g**: methyl paraben, **h**: 3-methylhentriacontane, **i**: pentacosane. The red boxes show simple CHCs, the green box highlights the chemical complexity of QMP. Figure adapted from (46)

The queen pheromones of *A. mellifera* honeybees include queen mandibular pheromone (QMP)(91), Dufour's gland extract (92) and tergal gland extract (93), as well as the CHC bouquet seen in other hymenopteran insects (47). Of these only Dufour's was unable to bring about reproductive repression of workers (94). Tergal gland extract (95), QMP (96), and CHCs (47), have all been shown to bring about reproductive repression of workers in honeybees.

That CHC profiles of honeybees were able to bring about reproductive repression in this species was only discovered very recently by comparing the CHC profiles of workers, virgin queens, and laying queens and exposing workers to artificial mixtures of these pheromones in order to investigate their effects on worker ovary development. The recency of this research highlights that there is still ongoing discovery of the nature and location of different queen pheromones (47).

1.3.1.1 Queen Mandibular Pheromone

QMP is a complex mixture of five main compounds: 9-oxo-2-decanoic acid (9-ODA), S/R 9-hydroxy-2-decanoic acid (2 optical isomers of 9-HDA), methyl paraben (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA) (97-100), with at least four other lesser components (101, 102).

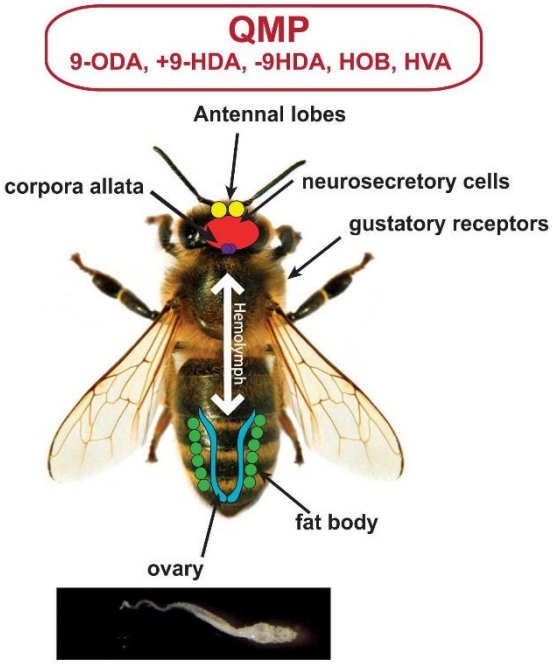


Figure 1.6 – Showing a diagram of possible QMP transduction from detection to ovary. Figure reproduced from (103, 104). In this model the pheromone is detected chemically via receptors, perhaps of the gustatory receptors in the mouth, gut, or legs, or of antennal receptors. The signal is transduced to the brain, where endocrine or neuroendocrine hormones are released to travel through the haemolymph directly to the ovary, or via the fat body first.

This queen pheromone is known to have repressive effects on the ovary activation of honeybee workers, however QMP also has a wide array of other roles affecting colony organisation. QMP induces care behaviour in the workers, such as grooming, nursing, and cleaning (105). It is also thought to be involved in successful swarming (106), repression of queen rearing (107), and repression of swarming behaviour (106). It also allows workers to identify the queen, and engage in retinue behaviours (108). In drones, QMP component 9-ODA acts as a sex pheromone, allowing the males to locate the queen for mating during mating flights (80).

The diverse set of roles of QMP have led to a large body of research investigating various elements of honeybee biology trying to characterise the mechanisms of action that this pheromone might be able to bring about these releaser and primer effects. Despite this

published research, there is still much that is unknown about the mechanism of action by which QMP brings about reproductive constraint of workers.

A reasonable, but as yet untested, assumption of this mechanism of action is that it is detected by olfaction/gustation, followed by signal transduction through the brain/nervous system/haemolymph, to bring about hormonal signalling to influence ovary development (possibly also via the fat body)(103). Detailed in Figure 1.6.

In this model, then, the mechanism of action of QMP can be split into three parts: its detection; the communication of this detection through the body of the bee; and the effect of that communication in the target tissues.

1.3.1.1.1 Detection of QMP

The odorant receptor Or11 has been suggested as a key detector for QMP, particularly for 9-ODA its role as a sex pheromone in drones (109, 110), however it must be noted that the detection method of drones is via true olfaction, i.e. detection of the volatile compounds via the antennae. Whereas workers require physical touch to bring about the repressive effect which QMP is able to bring about on worker reproduction (111).

Other work has highlighted an odorant binding protein which interacts with HOB, Antennal special protein 1 (Asp1) (112). This protein binds to the substance and allows it to traverse the aqueous layer for it to interact with nerve dendrons in the antennae.

These two examples of proteins which facilitate the detection of QMP components suggest that QMP is able to be detected at a neurological level, and therefore transduced into a signal in the brain.

Notably, it does not demonstrate that this is the mechanism by which QMP is able to enter the body of the bee for its role as a repressor of reproduction. It is a possibility that QMP is able to enter the bee directly, either through the cuticle, or via the gut, to bring about effect in the ovary itself.

There has yet to be identified receptor interaction with any of the other major or minor components of QMP which are known to bring about both primer and releaser effects in the honeybee, highlighting the significant lack of knowledge of signal detection in *A. mellifera*.

1.3.1.1.2 Transduction of QMP signal to the ovary

This model is also based on data showing that ecdysone (113), and juvenile hormone titres (114) change in the presence of QMP (with ecdysone reducing and juvenile hormone increasing). These hormones are incredibly important in the physiology of workers. It has also

been shown that QMP component HVA is responsible for changes in dopamine signalling in the brain (115). Dopamine levels in the haemolymph also vary a great deal between QMP exposed bees and those not exposed (104).

There are, therefore, significant changes in hormonal signalling throughout the bee in response to QMP. It is eminently possible that some, or all, of these signalling molecules and pathways are responsible for the transduction of the QMP signal from the neural system in the brain of the bee, through to the ovary, possibly via the fatbody. In particular, haemolymph titres of dopamine suggest that this neuroendocrine molecule may be the key mediator of this signal from brain to reproductive organs via diffusion through the haemolymph (104).

Again, there are alternative explanations to these level changes. In all cases, the ordering of signalling is not known. It could be that the ovary is responding to one, some, or all of these signals, but it could also be that ovary activation, and the fatbody changes associated with this, are responsible for the differences in somatic endocrine signalling that we see between bees exposed to QMP and those not, as it is known in insects that these tissues are also responsible for somatic endocrine signalling in some situations (116).

Another alternative explanation, is that these signalling changes occur as a consequence of the other roles of QMP, which include as repressor of queen rearing (107), trigger of care behaviour (105) and the associated physiological changes necessary for this (such as hypopharyngeal gland hypertrophy (117)), or its role in repressing swarming (106).

Whether the hormone changes are a consequence of QMP signalling directly, or whether they come about as a result of changes to the ovary and fatbody is a key question for contextualising the mechanism of action of QMP in this species.

1.3.1.1.3 The structure of the honeybee ovary

The honeybee ovary is a polytrophic meroistic ovary which differs in morphology between the worker and the queen castes (shown in Figure 1.7). In both, there exists two ovaries, each comprising a number of ovarioles, structured the same between the queen and the worker, however the worker ovarioles remain inactive under normal circumstances and so appear thin and threadlike. The ovariole number is sometimes asymmetrical between the left and right ovaries. (22, 49, 118, 119)

When active, however, the ovariole morphology is the same between the two castes. The number of ovarioles per ovary for workers can be as few as one, with as many as seven, however there are examples of workers with as many as 50. The queens possess ovariole

numbers in the 200 to 400 range, though this is also highly variable amongst queens, and amongst workers. (22, 49)

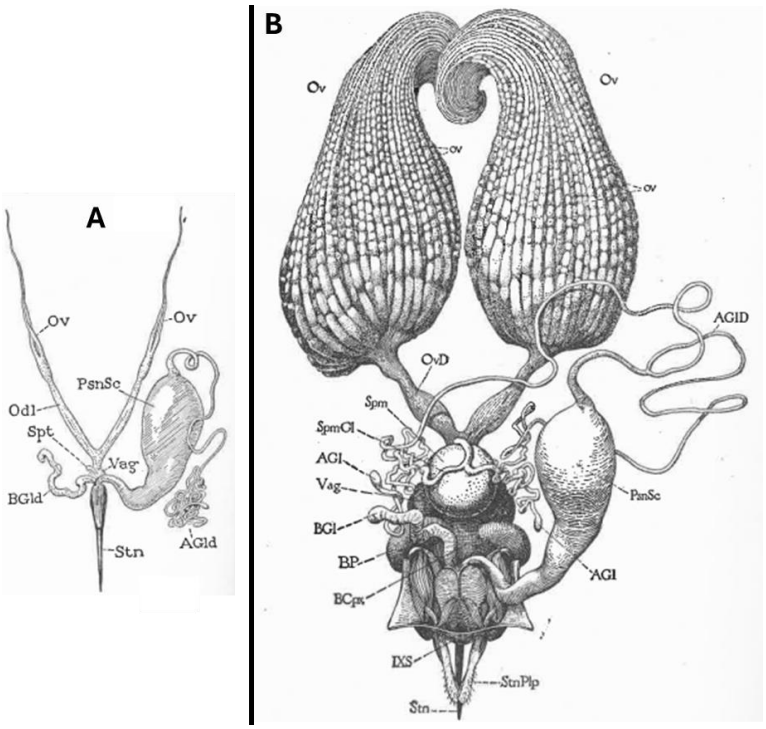


Figure 1.7 – Showing the difference in size and ovariole number between ovaries of honeybee workers and queens. In **A**, the inactive ovaries of a honeybee worker can be seen, with thin thread-like ovarioles of a small number feeding into the oviduct and vagina. In **B** the active ovaries of a queen can be seen with up to 200 hundred ovarioles per ovary. The accessory glands are also shown with the spermatheca and spermathecal glands in the queen ovary. Additionally, the sting, poison sac, and acid glands are also shown. Adapted from (22) and (49)

As detailed in Figure 1.8, the ovariole structure of the honeybee ovary is comprised of three sections, the terminal filament, the germarium, and the vitellarium. In the terminal filament, the oogonia (the precursors to both the nurse cells and the oocyte) reside. As these travel more towards the germarium, they begin to rapidly multiply and the follicle cells begin to differentiate. In the early part of the germarium, with the differentiation of the oogonia into the oocyte and nurse cell progenitors we begin to see the cystocyte cluster beginning to form. This cluster contains a single oocyte, bordered by follicle cells, with several nurse cells attached to the rear of the oocyte, which advances into the vitellarium. The nurse cells provide various cytoplasmic components to the developing oocyte such as mRNA, mitochondria, and

ribosomes, as well as nutritive elements such as lipids(120-122) in order to more rapidly advance its development. (22, 49, 118, 119, 123)

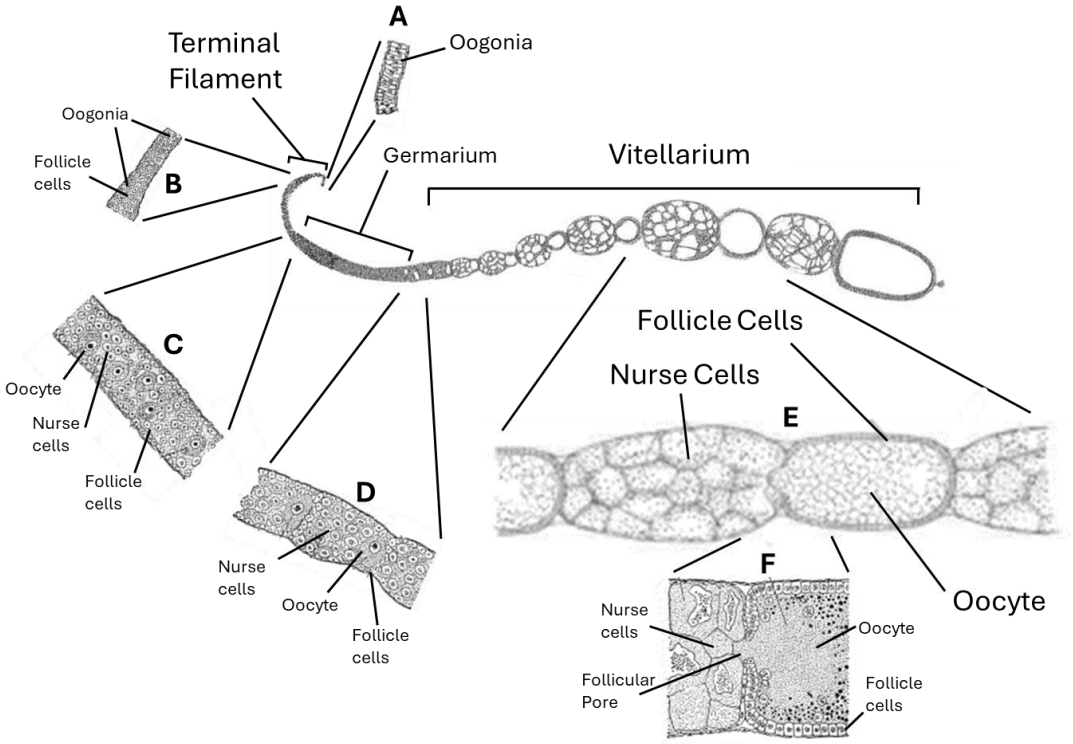


Figure 1.8 – Showing the structure of an active honeybee ovariole. A honeybee ovariole is divided into three parts: the Terminal filament, where the oogenic stem cells reside; the Germarium where oogonia transition into the oocyte, nurse cells, and follicle cells; and the Vitellarium, where the developing oocyte matures into an egg. **A** shows the tip of the terminal filament with undifferentiated oogonia. **B** shows the terminal filament further down where oogonia are multiplying and follicle cells begin to appear. **C** shows the start of the germarium where oogonia have begun to differentiate into the oocyte and nurse cells, with follicle cells forming a layer inside the epithelium of the ovariole. **D** shows the end of the germarium where the oocyte, nurse cells, and follicle cells have begun to assume their final orientations before continuing into the Vitellarium. **E** shows the cystocyte, a collection of nurse cells in conjunction with a developing oocyte and its surrounding follicle cells. **F** shows a close up of the follicular pore which allows for RNA to pass from the nurse cells into the developing oocyte. Adapted from (123), (49), and (22)

In addition to the resources provided by the nurse cells, the follicle cells, which act as the outer sheath of the cystocyte, provide nutrition to the developing oocyte in the form of vitellogenin, which is synthesised in the fat body (124), before being taken up by the follicle cells and transferred into the cystocyte (125).

Throughout the vitellarium, the oocyte continues to grow alongside the nurse cells, until towards the end, where the nurse cells begin to fully dump their cell contents into the developing oocyte. By the end of the vitellarium, the nurse cells have completely disappeared, and the oocyte has developed a chorion outer sheath, becoming an egg ready to be laid, which it then subsequently is, into a cell in the comb of the colony, or otherwise resorbed if this isn't possible (126, 127). (22, 49, 118, 119, 123)

1.3.1.1.4 The Ranking of Ovarian Development

In order to accurately report the reproductive activity of insects, various metrics have been developed. The simplest is to count the number of eggs which the individual lays (e.g. (128)), however this is an ineffective method for measuring ovary development in insects such as the honeybee. In this species ovary activation is not necessarily linked to egg-laying behaviours, and social policing, whereby workers will consume eggs laid by other workers, obfuscates metrics (129).

A more direct measurement used to investigate ovary activity in hymenopteran insects is a form of Hess score (130), originally coined for use in honeybees, but adapted into bumblebees (131). In this system, qualitative developmental milestones are observed and used to rank the ovary development, usually into one of four categories. In honeybees for example (132), the ovaries are scored 0-3: where 0 represents no visible cell differentiation; 1 represents observable cystocyte formation; 2 represents observable yolk deposition; and 3 represents fully developed oocytes visible (see section 2.6.1.1 for more detail).

Other ways of measuring ovary activity are to measure certain aspects of the ovary, e.g. in the yellowjacket wasp *V. vulgaris*, an inactive versus an active ovary is categorised by comparing the largest oocyte of an ovariole to a fully developed egg (133). If the largest oocyte is larger than half the size of an oocyte the ovary is considered active, otherwise it is considered inactive (133).(133) Or to produce a quantitative measure of ovary activation such as in *D. melanogaster*, where the number of developed eggs in an ovary can be measured (134), or the area of the ovaries when imaged (135).A more developmentally rooted form of ovary scoring, such as the Hess score, allows for more direct comparisons of ovary activity and ovary development. By linking the ovary score to specific developmental stages, such as between 0 and 1 in the honeybee Hess score, which equates to the germarium/vitellarium boundary, or

between 1 and 2 which represents the onset of yolk deposition; we can make attempts to link the ovary activity of the organism back to its sexual development.

1.3.1.1.5 Effect of QMP signal in the ovary

Although various ovarian checkpoints (whereby the developing oogonia are arrested in their development at specific points in that development as a mechanism of control over reproduction) have been identified in model organisms such as *D. melanogaster* (136), the ovarian checkpoints of honeybees are less clear (137).

It is known that QMP exposure results in an increase in the activity of the Notch receptor signalling pathway in the germarium of honeybee workers, but not of the ligands of the Notch receptor; additionally an inhibitor of Notch eliminates QMP-mediated reproductive constraint (132). This quasi-paracrine signalling method involves direct inter-cellular contact (138), and so perhaps represents a signal amplifier in this tissue i.e. it is able to take a signal from some other source (e.g. such as insulin or dopamine from the haemolymph) and amplify it within the tissue to produce an increased local response. This possibly indicates that there is a germarium-linked ovarian checkpoint in honeybees.

Additionally, by comparing queenright workers to queens, Tanaka et al. showed using histological analysis that the terminal filament and early germarium of both castes are functionally identical. Only in the transition to early cystocyte cluster formation are differences observed, with evidence of cell death (139), possibly indicating that this is where a putative checkpoint is located within the ovariole.

The role of Notch in the germarium (132), as well as histological evidence investigating the cell types in queenright workers (139), provides good evidence for the early-to-mid germarium being the sight of an ovarian checkpoint. This would reflect the transition from Hess score 0 to 1 (132), however this is not very clearly observable in practice due to the difficulty in observing these cell types with traditional microscopy.

Additional evidence for a mid-oogenesis checkpoint (in the late germarium) is provided by Ronai et al. in the form of PMP34 (aka *Anarchy* (140, 141)). In this paper, PMP34 was chosen for investigation first via comparison of gene expression profiles between ovaries of workers with active and inactive ovaries (142). It was chosen as one of four genes for investigation based on microarray analysis published by Thompson et al. (143), from the same lab group, and is associated with a region associated with the anarchic honeybee phenotype (whereby workers are able to lay eggs even in the presence of a queen) (142), also from the same lab group. The low number of target genes is surprising due to the fact that more recent research

using high throughput RNA sequencing found more than 5000 differentially expressed genes in the ovary between inactive and active ovaries of workers (144).

There are also significant methodological issues with this research. The two techniques used to link PMP34 to apoptosis, and subsequently to the ovary as a proposed ovarian checkpoint are: dsRNA injected directly into the abdomen of worker honeybees in order to knockdown expression of PMP34 in the abdomen of the worker; and mFISH to localise this effect to the ovary. In the former technique the effect is demonstrated to be transient within 24 h of injection with no data on subsequent ovary activity and mortality, casting doubt on their conclusion that this is central to ovary repression, and indeed it has been shown that RNAi injections into honeybee abdomens are taken up almost exclusively by the fatbody (145). The only evidence for localisation to the ovary comes from the *in-situ* hybridisation, which was carried out on ovaries which had been freeze-thawed twice before hybridisation, which invalidates the protocol by allowing the mRNA to permeate the tissue (146). As a result, this study, and those based on it, are unlikely to represent the mechanisms of a mid-oogenesis ovary checkpoint.

Despite the fact that this proof of PMP34 as the mediator of the early ovarian checkpoint is suspect, it is likely that this checkpoint does exist, likely mediated (at least in part) by QMP exposure in worker honeybees. This provides a simple example of how QMP may be repressing the reproduction in the worker caste of *A. mellifera*, however it is not clear if this is the only such example of a checkpoint in the ovary, nor how this signal is induced in this tissue.

There are still many gaps in our understanding of the role of QMP at every stage in its mechanism within the honeybee, highlighting the need for research to clarify the mechanism of action of QMP-mediated reproductive constraint in *A. mellifera*, particularly with how this relates to repression of oogenesis in the ovary of this species.

1.3.1.1.6 Broad Activity of QMP

Unusually, QMP is incredibly wide acting across the animal kingdom (46). I.e. it can bring about reproductive repression in evolutionarily adjacent species, such as the bumblebee *B. terrestris* (128), which shares an origin of eusociality with honeybees (147). But it can also bring about inhibition of reproduction in the fruit fly *D. melanogaster* (148), and even as distantly as a prawn: *Leander serratus* (149). The wideness of effect can be seen in Figure 1.9 (in section 1.5), though in this figure neither *Osmia*, nor humans have been shown to have reproductive repression induced by QMP (123).

The wide-acting nature of QMP seems to be unique amongst the queen pheromones of hymenopteran insects, whose CHC-based queen pheromones generally display a small amount of cross-activity in closely related species, but not widely across the insects (46, 134).

Although QMP is able to act widely, it is not universally repressive, for example it has recently been shown to be unable to bring about repression in the social wasp *V. vulgaris* (133). This might be as a result of inappropriate use of concentration (as acknowledged in the paper itself), however it could also be a result of the coopting of one of the detection, transduction, or effector elements of the pathways which are used for QMP-mediated repression of ovaries in the other species which QMP is able to act in. The reproductive ground plan hypothesis suggests that the regulatory networks governing maternal care and reproduction have been decoupled in eusocial insects (66, 67, 150), but it does not have to have occurred the same way in all species. The fact that *V. vulgaris* does not share a common origin of eusociality as the honeybees and bumblebees (7), possibly suggests that the loss of QMP-sensitivity is as a result of its own eusocial evolution.

1.3.2 Larval pheromones

In eusocial insects, the nature of cooperative brood care necessitates a degree of communication between the developing brood and their caretakers. These are called larval, or brood pheromones (151).

In the honeybees, these pheromones have been linked to several different effects in both *A. mellifera* and *A. cerana* (the Asian honeybee). The best understood is the releaser feeding response, whereby the larvae will produce a pheromone to differentiate themselves from pupae, and thus attract workers in order to be fed, which seems to be fairly a common response to brood pheromones across the eusocial hymenopterans. (152-155)

However larval pheromones have been linked to several primer effects too, including influencing the ratio of nurse workers in colonies (156), influencing longevity of workers in the context of the colony (157), affecting the rate of pollen foraging (155), and aiding in the reproductive constraint of workers (158).

In *A. mellifera*, there have been several identified components of a pheromone labelled Brood Ester Pheromone (also called Brood Pheromone, BP): 10 fatty acid esters (159), which have wide-ranging effects within the colony. A second larval pheromone E-β-Ocimene has also been discovered, posited as related to social regulation in the honeybees (160), including visitation of cells (161), but has also been linked to ovary repression in the adult workers (162).

BP has several known effects on *A. mellifera* colonies, thought to be caused by subsets of the 10 components: BP has been shown to influence the ratio of pollen-foragers, to non-pollen foragers (163); the number of foragers total (by decreasing the minimum age at which workers start to forage, as in honeybees age is highly correlated with job role amongst workers) (44); the overall number of brood in a colony; the frequency of foraging flights (163); the average load of pollen (164); and longevity in workers (157) (putatively related to the transition to over-wintering behaviour) (165).

In addition to these known effects, there is evidence to suggest that some of the components (putatively methyl linolenate and ethyl palmitate (158)) are responsible, at least in part, for ovary repression in worker bees (154). Mohammedi et al. (158) showed that when exposed to the individual BP components *in cavea*, queenless honeybee workers were reproductively repressed only by these two BP components when individually applied to worker honeybees, though it is noted that there was non-statistically significant repression with the other components. This was a follow up study to that published by the same lab group, demonstrating that BP collectively was able to repress worker reproduction under similar conditions (166).

The reproductive constraint aspect of BP and EBO in honeybees provides evidence for yet another redundant mechanism through which reproductive constraint in this organism is maintained (47). However, the mechanism of action through which these pheromones are able to bring about repression of worker reproduction is entirely unknown. Their detection, signal transduction, and effect have not been investigated in any other way than observations of ovary development with and without exposure. There exists therefore, many gaps in our understanding of these pheromones.

1.4 Honest Signal of Fertility or Reproductive Constraint

An ongoing conversation in the field is whether queen pheromones evolved to forcefully repress the reproduction of workers against their own interest, or whether these pheromones act as honest signals of the queen's fertility to the workers so that they can identify the reproductive fitness of the colony and maintain social organisation as a result (167).

In many eusocial hymenopteran insect species, workers often have the ability to lay unfertilised eggs which are able to be reared to adulthood as males (168). There is a possibility, therefore, of direct fitness of an ordinarily sterile worker. Under normal social conditions however, the queen, by virtue of her superior reproductive ability, the greater relatedness of the worker to

her sisters, and the need to maintain optimal sex ratios, is a better conduit through which a worker can exhibit their reproductive fitness (20).

Hamilton's rule suggests, therefore, that as long as the social conditions are maintained, and the queen retains her fertility, it is in the workers individual interest to maintain their position in the colony (60, 167).

It is in the interest of the worker then to have an indicator of whether the queen is maintaining her fertility, and it is in the interest of the queen for the worker to believe that the queen maintains her fertility. It is proposed that queen pheromone exhibits its function as an honest signal, and that this is the primary evolutionary origin of these pheromones (167).

Alternatively, in an ancestral state in which adjacent individuals in a facultatively social, or primitively eusocial colony, do not morphologically differ and who engage in cooperative brood care, it is in the interest of individuals to be able to repress the reproduction of the others in the colony in order to maintain their own direct fitness due to this increasing proportion of care which is provided to their own brood by the others around them (This is balanced by the indirect fitness which the repressing individual loses if there is close relatedness between these cooperating individuals. In such a situation, the individual doing the repressing would have to balance their own fitness against the loss of fitness of their kin, and so this selection pressure would also correlate strongly with reproductive potential of the repressor). It is in the interest of the others to resist this control. If the signal is linked to reproductive activity, then larger ovaries and greater fertility results in greater repression of others. Therein lies the argument that queen pheromones instigate reproductive constraint against the interest of the workers in a eusocial context (132).

There is evidence for both theories, potentially indicating that the two are not mutually exclusive with regards to the current state of eusocial control, however the evolution of these pheromones would be different according to each theory. The lack of diversity and inter-species activity of CHC queen pheromones, implies that most species have evolved with queen pheromones acting as honest signals of fertility, such as in the bumblebee *B. terrestris*. However, the diversity and complexity of QMP could very well have come about as a result of an arms-race style evolutionary battle between a queen attempting to overcome the reproduction of the workers, and the workers developing resistances to the pheromone (169), indeed this is observed in the Cape honeybee subspecies *Apis mellifera capensis* (170, 171). Additionally, QMP component concentration does not correlate with ovary size (172), though notably it does differ between mated and unmated queens (173), perhaps suggesting that QMP is acting as an honest signal of fertility with regards to mating status rather than absolute

fertility. Other research suggests that QMP acts as an honest signal of fertility via its role in repression of queen rearing (107). In this paper, QMP provision (alongside the presence of brood) prevented the development of new queens. If QMP was only acting as a repression of reproduction against the interest of the workers, then it would be expected that workers would attempt to lay their own eggs after the removal of QMP-induced constraint. The fact that they instead attempt to rear a new queen is evidence of QMP acting as an honest signal of fertility.

1.4.1 Sender-Precursor and Sensory Exploitation

The evolution of queen pheromones as honest signals of fertility to workers, or as forceful agents of reproductive repression against the interest of the workers, is influenced by the nature of the evolution of these compounds. The two mechanisms by which such a pheromone might evolve depends upon both the nature of the compound and its ability to affect a response in a respective receptor. These two methods of evolution are the sender-precursor hypothesis and the sensory exploitation hypothesis.

The sender-precursor hypothesis is that both a signal and its receptor had ancestrally alternative functions that coevolved over time together to be coopted into the function we now observe. This contrasts with the sensory exploitation hypothesis, which states that the signal evolved to take advantage of an important, but otherwise unrelated pathway to give it a new function which we now observe. (46, 89, 174, 175)

The CHC-based queen pheromones of the majority of social hymenopterans are perhaps evidence of the sender-precursor hypothesis. The ancestral role of CHCs is as anti-desiccants and nest-mate recognition signals, which could have evolved into queen pheromones as specific bouquets are produced with greater fertility. In this manner, the signal acts as an honest signal of fertility.

The QMP of honeybees however, which is able to act far more widely than the CHCs of the other hymenopterans, represents a potential example of sensory exploitation. With the possible co-option of e.g. Notch signalling in the ovaries (132), or dopamine signalling via the haemolymph (104), and taking into account the reproductive ground plan hypothesis; we can see the same reproductive repression in the fruit fly as in the honeybee. This wide repression could have come about as a result of the honeybee queens serendipitously evolving to produce a queen pheromone by sensory exploitation to take advantage of these fundamental mechanisms in order to repress the reproduction of their workers against the better interest of those workers.

1.4.2 Reproductive diapause

Insect diapause is a state in which metabolic, physiological, and anatomical changes to the individual occur, in order to maximise fitness in response to environmental stress.

The terminology used for these states is relatively broad, and inconsistent between species. For example in *D. melanogaster* fruit flies, the adult reproductive diapause is often called reproductive dormancy (103) and vice versa.

Diapause is perhaps best characterised in insects which undergo a form of diapause-hibernation during winter due to lowering temperatures (e.g. such as the solitary bee *O. bicornis* (176) or the social wasps *Vespula* (177)), though it is also well studied in relation to larval, or pupal diapause, which results in holometabolous insects delaying, or halting metamorphosis in order to wait for more optimal environmental conditions for reproducing (178, 179). This is particularly true for those species who only enter adulthood to mate, and only stay in adult for a very short period of time (such as the mayfly (180)).

In adults, diapause generally allows for the maximisation of direct fitness by stopping reproduction from occurring in periods of inopportunity. By delaying reproduction (and therefore increasing lifespan in line with the fecundity-longevity trade off (181)), and instead devoting metabolic resources to survival, such as the upregulation of CyP450s to deal with chemical stress (182), the individual is able to better survive the short-term stress being imposed upon them, and so maximise their own fitness in the long term.

It has been suggested that QMP-mediated reproductive constraint is able to occur in honeybees as a result of the co-option of starvation-induced diapause mechanisms (179), and that this co-option represents a sensory exploitation that allows it to work in other, distantly related, insect species (103). This is based on work produced in fruit flies suggesting that QMP acts via inducing starvation in this species, and consistent with the sensory exploitation hypothesis, that this is potentially the same mechanism in the honeybee (183). It is also based on a comparison of the similar endocrine responses of reproductively inactive fruit flies and honeybees whose reproduction has been repressed by QMP (113, 114, 184, 185). Additionally, the role of stress in the QMP-mediated reproductive repression in honeybees has also been highlighted as a potential mechanism, and this is in line with the idea of diapause being the mediator (103).

1.4.2.1 Diapause in Honeybees

Honeybees possess one predominant type of adult diapause: the winter bee phenotype as a longer term diapause in which honeybee queens cease reproducing (186). This is triggered by the onset of Winter, possibly as a result of the lack of pollen stores (187).

In the winter bee form, most hive roles change, with no foragers, reduced cleaning, and due to lack of brood no nursing behaviour either. In this form, the bees form a ball-shaped cluster of bees around the queen, located on comb filled with stores from the prior summer (188). The longevity of workers also greatly increases, with lifespan tripling during the colder months of the year (189). During this period workers will not activate their ovaries, and the queen's ovaries also regress (190).

The behaviour of the bees is such that they are able to maintain the internal temperature of the hive at a consistent 35°C for the entirety of the winter, even when external temperatures are far lower than this. It has been demonstrated that bees are able to maintain this internal temperature when the external temperature is consistently as low as -20°C (52), though honeybees have also been shown to have been able to survive temperatures as low as -28°C (191). Notably the cold-tolerance of honeybees is highly variable with strain, for example the native British Black *A. mellifera mellifera* is known for greater cold tolerance than the more commonly used Italian *A. mellifera ligustica* (192).

There is a second form of colony diapause, in which workers will consume young larvae under periods of pollen-stress (such as during drought, or particularly prolonged wet weather). However there does not seem to be a reduction in oviposition during these periods, and so is likely not a true diapause, and is mischaracterised as such (193, 194).

1.4.2.2 Diapause in Fruit Flies

Diapause in *D. melanogaster*, often called reproductive dormancy in this species, is relatively well characterised (195-197). It can be reliably investigated in a lab setting via the use of cold and shortened day light cycle (198). There is dispute over whether the quiescence seen in this species truly represents diapause, as the diapause effect is relatively short-lived and the species quickly adapts away from it if given the opportunity (199, 200). However, for the sake of this work they will be treated the same, as there is solid evidence to that effect (201-203).

In the fruit fly diapause is associated with inhibited ovary activity, increased insulin signalling, longer life-span, upregulation of genes involved in innate immunity, lower food consumption, decreased senescence, and increased stress resistance. (199, 200, 204)

Fruit flies possess a very high plasticity with which they enter and exit reproductive diapause in response to the environmental conditions which trigger it. I.e. in periods during which they are required to go into diapause more frequently, such as during winter, they become potentiated to entering diapause more rapidly, and this maintains in the months of spring.(199)

1.5 Study Organisms

Throughout the course of this thesis, three principle study organisms are the basis of investigation: the Western honeybee *A. mellifera*, the fruit fly *D. melanogaster*, and the buff-tailed bumblebee *B. terrestris*. In addition to these organisms, the biology of several others were used to inform the experimental design of the species used therein (such as the solitary bee *O. bicornis*). The phylogenetic relationship of these organisms can be seen in Figure 1.9.

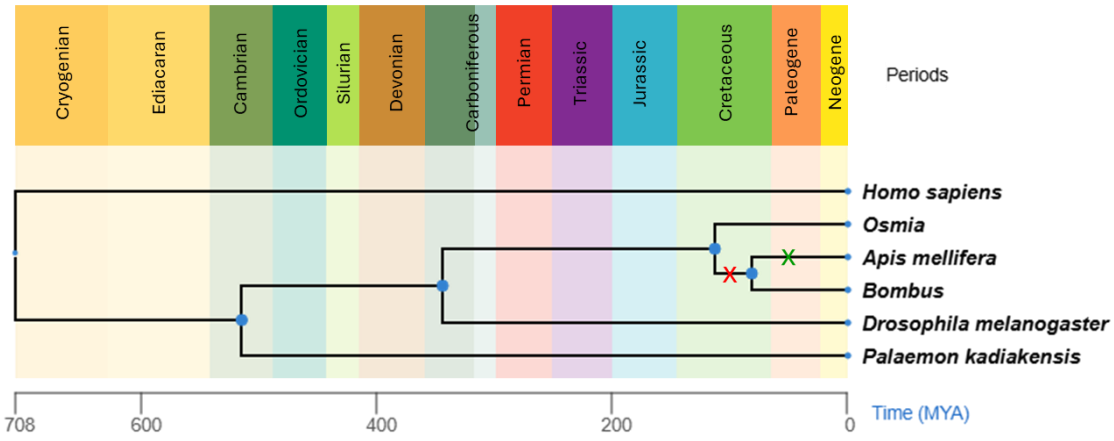


Figure 1.9 – Showing the phylogenetic relationship of the models used in this thesis. The time point of the last common ancestor between two species is given as a branch, with the millions of years ago (mya) given along the bottom. The geological periods are given along the top, with the species shown on the right. The red cross represents the evolution of eusociality in the bees, and the green cross represents the evolution of QMP. Humans, *Homo sapiens* are given as an outgroup, with the shrimp *Palaemon kadiakensis* given as a member of the genus of shrimp in which QMP is able to induce reproductive repression. The genus *Osmia* is shown not to respond to QMP, while *A. mellifera*, *Bombus* genus, *D. melanogaster* and *P. kadiakensis* have shown to be reproductively repressed by QMP.

1.5.1 *A. mellifera* honeybee

The *A. mellifera* honeybee has been one of the most culturally, historically, and economically important insects throughout human history. Having been domesticated and cultivated since at least 8000 BC (205), *A. mellifera* has been kept for its wax, larvae, pollen, and honey (the only source of pure sugar in antiquity). Today the beekeeping industry also includes the pollination

services for a significant proportion of fruit, vegetable, and nut agriculture, particularly in monocultured agricultural systems. In some parts of Africa and Asia, the African honeybee *A. mellifera scutellata*, has also been used as a defence system for farms against wildlife, particularly elephants (206). Honeybees exhibit a highly derived form of eusociality, making them particularly relevant as a study organism.

As laid out in section 1.3.1.1, the mechanism of action of pheromonally induced reproductive constraint is relatively poorly understood in this organism at a physiological level, and so forms the basis of a significant proportion of the research presented herein.

1.5.2 *B. terrestris* bumblebee

A sister species to the honeybee is the bumblebee *B. terrestris audax*. One of 27 species of bumblebee in the UK (207), it is particularly common in urban environments with its characteristic two yellow bands and buff-coloured tail. It is an annual primitively eusocial species, which is used widely as a greenhouse pollinator: commercially available colonies can be placed in industrial greenhouses to pollinate crops such as bell peppers, in order to increase yields (208). Bumblebees are the closest extant bee species to the honeybees, sharing an origin of eusociality and having separated relatively recently, at around 30 mya (7).

B. terrestris has been shown to be sensitive to QMP-mediated reproductive constraint (209), as well as possessing its own queen pheromone (46, 88).

The relatively close evolutionary relationship between this species and the honeybee, as well as the single origin of eusociality, makes *B. terrestris* a useful study species for investigating the evolution of pheromones in honeybees. By comparing and contrasting the mechanisms and phenotypes of these pheromones in both species, we can infer important clues as to the nature of the evolution of these pheromones and these species.

1.5.3 *D. melanogaster* fruit fly

D. melanogaster has been a common model organism since the early 20th century. There are a variety of advanced molecular and genetic techniques which can be used to investigate the biology of this species as a result, such as the UAS-Gal4 system. That it is also responsive to QMP-mediated reproductive constraint, allows us to use these advanced techniques to investigate the mechanism of QMP in this species, in order to compare it to the other species in which QMP is able to act.

We can then elucidate whether sensory exploitation is the mechanism by which QMP is able to evolve, the role of diapause in the process, and whether the mechanisms are proximate between species.

1.6 Note on Nomenclature

The majority of the bee research conducted in this thesis involved the removal of honeybees and bumblebees from colonies into metal cages. The biological differences between these two environments is akin to *in vivo* versus *in vitro* studies, however this nomenclature is not appropriate for this setting due to the *in vivo* nature of all of the experiments.

As a result, throughout this thesis the words *in cavea* will be used to refer to “in cage” settings, and *in alvo* will be used to refer to “in hive” settings. This nomenclature allows us to distinguish between a more natural *in alvo* setting, and a less natural but more sterile *in cavea* setting, without inappropriately co-opting existing terminology in order to force the intention.

1.7 Research Aims

The primary aim of the research presented in this thesis was to elucidate the mechanisms by which pheromones are able to bring about reproductive constraint in eusocial insects, and how this has come to evolve.

The principal pheromone investigated was the honeybee queen pheromone QMP. As it is broad active in its reproductive repression across different species of insects, it allows us to investigate the evolutionary aspects of eusocial evolution in honeybees, particularly as the precise mechanisms through which it is able to bring about reproductive repression are not very well characterised, nor whether it is working via the same mechanisms in each of the species used in this thesis. The mechanism of its action in honeybees was investigated in Chapter 3, of bumblebees in Chapter 4, and of fruit flies in Chapter 6.

The roles of other pheromones: BP, EBO, and nC25, as well as the role of the plant polyphenol quercetin, were investigated in bumblebees in Chapter 4, and in honeybees and fruit flies in Chapter 5. This was to lay the foundational ground work for investigating the mechanism of action of these pheromones, which is, as yet, uncharacterised.

Specifically, in Chapter 3, in honeybees, I investigated the mechanism of action of QMP by investigating the effect of QMP on feeding, mortality, and ovary activation. I also tested the plasticity of honeybees to QMP-mediated reproductive repression; the temporal limits of QMP-mediated repression; and the sequence of signalling which occurs in response to QMP exposure after eclosure from the pupae.

In Chapter 4, I established a bumblebee model and use it to investigate the mechanism of action of pentacosane and QMP in this species, as neither pheromone has yet been characterised for its mechanism of action in the literature.

In Chapter 5, I investigated the effect of BP and EBO in fruit flies in order to determine if honeybee brood pheromones are as widely acting as QMP. I also investigated the effect of these pheromones in honeybees in order to investigate their mechanism of action, as this is completely uncharacterised with regards to its ability to bring about reproductive repression.

In Chapter 6, I investigated the mechanism of action of QMP in fruit flies in order to investigate the hypothesis that diapause is a coopted biological response through which QMP is able to bring about reproductive repression.

In Chapter 7, I drew together the data presented in this thesis to contextualise the findings in the literature, as to the nature of pheromonally mediated reproductive constraint.

Chapter 2 General Methods

2.1 Ethics

In the UK, ethical approval is not required for any research involving live insects (210). There is, however, a growing body of research suggesting that insects, and bees in particular, are possessing of far higher levels of awareness and sensory experience than assumed in the 20th century, including the ability for nociception without the presence of identifiable nociceptors (211-213).

Ethical guidelines for the research contained herein were implemented accordingly, ignoring the lack of such guidelines at a legal framework level. It is also hoped that these methods can be built and developed upon further into a framework which minimises harm to research organisms.

In accordance with this, all dissections and tissue collections were conducted on fully anaesthetised individuals. If the individuals began to wake up during the dissections, efforts were made to euthanise immediately (for example by crushing the thoraces of fruit flies). All excess heads and thoraces of honeybees and bumblebees from ovary dissections were placed in cold soapy water before they awoke in order to euthanise them. Occasionally bees would still wake up in these conditions, whereupon their heads and thoraces were crushed with forceps to euthanise.

All bees not used for experiments were either returned *in alvo* where possible or euthanised by freezing at -20°C for honeybees, or at -80°C for bumblebees (as bumblebees take much longer to euthanise at -20°C). Escaped bees which could not be returned *in cavea* or *in alvo* during the course of experiments, might also have been crushed in order to minimise stress to the bee or harm to other researchers in the lab environment, where capture and freezing was inappropriate.

In cavea bees were kept in the dark where possible to minimise stress, with as limited physical disturbance as possible. *In alvo* humane beekeeping practice was observed at all times, including limited contact during the winter.

2.2 Reagents

2.2.1 Pheromones

2.2.1.1 Queen Mandibular Pheromone

Artificial Queen Mandibular Pheromone (QMP) (Intko Supply Ltd, Canada), was supplied in a vial of wax weighing 0.5 g for 1182 Queen equivalents (Qe) of QMP (with one Qe being the amount of QMP produced by a single queen in a day (214)), dissolved in 504.8 µl of ethanol to produce a stock of 26Qe/20 µl from which working solutions were obtained. Components of this 0.5g wax are shown in Table 1. The wax was stored at 4°C, and the ethanol-dissolved stocks were stored at -20°C. For honeybee work, at the start of each summer a new vial was made up in order to mitigate any potential degradation of QMP efficacy over long-term storage, and each new vial was validated with a simple QMP exposure against ethanol control (see section 2.4.2.1). Additionally, for any *D. melanogaster* experiment using large quantities of QMP (such as the QMP-dose response in section 5.3.2.1, or the QMP exposures of Chapter 6), a fresh vial was made up, and likewise validated via a simple QMP exposure against ethanol control (see section 2.4.1.2).

Table 1 – Showing the relative composition of QMP (214)

Component	Mass /mg
9-ODA: (E)-9-oxodec-2-enoic acid	261.8
9-HAD-R: (E)-9-hydroxydec-2-enoic acid	104.7
9-HAD-S: (E)-9-hydroxydec-2-enoic acid	104.7
HOB: methyl p-hydroxybenzoate	26.18
HVA: 4-hydroxy-3-methoxyphenylethanol	2.62

N.B. this is different to previously published concentrations of QMP in (111, 215-217), which had not taken into account the volume displacement of wax when calculating ethanol volume and so have recorded their QMP concentrations as more concentrated than they are in practice, their recorded QMP concentrations are about 1.4x the true concentrations (i.e. that the concentration used was less concentrated than reported).

2.2.1.2 Pentacosane

Pentacosane (n-C25)(Sigma-Aldrich) was dissolved in pentane to produce a 16 Qe master stock (with one Qe being 232.5 µg, the amount of pentacosane produced by a single *B. terrestris* queen in one day (46)), the stock was stored at -20°C.

2.2.2 Buffers

2.2.2.1 Phosphate Buffer Saline

Phosphate Buffer Solution (PBS) was made up as a 10x stock in double distilled water (ddH_2O). The 10xPBS was adjusted to a pH of 7.4 using NaOH or HCl. For use, the 10x stock was diluted 1:10 into ddH_2O. Composition in Table 2

Table 2 – Showing the composition of 10x PBS

Solute	Concentration (g/L) for 10x
NaCl	102.2
NaH_2PO_4	2.56
Na_2HPO_4	11.94

2.2.2.2 PTx permeating buffer

PTx is comprised of Triton (Sigma) diluted in PBS at 0.1x, i.e. 50 µl of Triton X-100 into 50 ml of PBS.

2.2.3 Food Recipes

2.2.3.1 *A. mellifera* Honeybee

2.2.3.1.1 Complete Bee Food

Complete Bee Food (CBF) was made up as in Table 3, and stored as a powder at -20°C. When used it was mixed with raw honey (sourced either from the supermarket or extracted from the hives in the apiary) till a dough-like consistency.

Table 3 – Showing the composition of CBF

Reagent	Mass /g
Pollen (LiveMoor)	20
Sugar (Tesco)	52
Brewers yeast (Thermo Scientific)	18.8
Lactalbumin (Gibco)	9.2

2.2.3.1.2 Fondant and Pollen diet (FandP)

During the course of *in cavea* honeybee experiments it became necessary to develop a more biologically realistic diet which could increase the lifespan of workers *in cavea* beyond the maximum 10-12 days which CBF is able to produce. It was noted that with a CBF diet, the workers had excessively distended abdomens when they were found dead, and during dissections on day 10, the gut was observed to be swollen with partially digested CBF. As a result, the FandP diet was developed.

Table sugar (Tesco) and pollen (LiveMoor) were separately ground in a coffee grinder until a fine powder, these were then stored at -20°C until used. For use the powders were mixed with raw honey at a ratio of 7:3 pollen:honey, and 3:1 sugar:honey.

This diet was able to lengthen lifespan *in cavea* to a maximum of 20 days and avoided the previously observed CBF issues.

2.2.3.2 *D. melanogaster* Fruit Fly

2.2.3.2.1 Bloomington Cornmeal Media

Cornmeal media was made by dissolving agar in 1 l of tap water followed by adding 2 l of tap water with cornmeal, yeast, and sugar mixed in. The mixture is cooked until the correct consistency, then taken off the heat before adding propionic acid and nipagin. The whole mixture is then poured in 7 ml aliquots into 15ml fly vials and left covered for 24h before being corked with cotton wool and stored at 4°C until use. For use the vials were warmed to room temperature first. The amounts of each component are in Table 4.

Table 4 – Showing the composition of Bloomington cornmeal fly flood

Reagent	Amount
Agar	27 g
Cornmeal	200 g
Yeast (Thermo Scientific)	50 g
Sugar (Tesco)	140 g
Propionic acid	20ml
Nipagin (10% w/v/ in ethanol)	20ml

2.2.3.2.2 Liquid Fly Food

Liquid fly food was made fresh on the day of use according to the proportions found in Table 5. It was dispensed in 500 µl doses, one per vial.

Table 5 – Showing the composition of liquid fly food

Reagent	Amount
Water	4.75 ml
Ethanol	0.25 ml
Sugar	0.15 g
Yeast	0.1 g

2.3 Animal Husbandry

2.3.1 *A. mellifera* Honeybees

Several hives of ordinary apicultural bees (putatively a mixture of the subspecies *A. mellifera ligustica, A. mellifera carnica*, and *A. mellifera mellifera*), were maintained in national-type polystyrene hives on campus at the University of Leeds. The hives were treated annually against *Varroa* mites by dribbling oxalic acid (3.2% w/v in 30% w/v sucrose in water) onto each frame of bees as they cluster in mid-winter, typically in mid January. For experiments, frames of brood were taken from stronger hives, with rotation so as not to stress one individual hive too

much. For swarm control, ordinary beekeeping methods were used, such as destruction of swarm cells or splitting where necessary. During superseding or post-swarm, hives were left for >3 weeks to recover. Occasionally it was necessary to replace queens from local beekeepers due to loss of hives over winter, or to replace aggressive hives. Honey was occasionally taken from the most productive hives and stored for use in experiments as a feed. Hives were fed sugar fondant (BeeCandee, Beekeeping Supplies UK) over winter and in the spring pollen mixture (ApiCandy, Beekeeping Supplies UK).

2.3.2 *Bombus terrestris* bumblebees

Agricultural *Bombus terrestris audax* bumblebee colonies were obtained from Agralan LTD (www.agralan.co.uk) as required. They were fed Biogluc

(Agralan) and pollen (LiveMoor) *ad libitum*. The Biogluc contains sugars and essential amino acids, while the pollen is a protein source. The colonies were kept inside their cardboard box covers in darkness at 27°C in a constant temperature room, when opening colonies to extract bees for experiments, a pure redlight LED was used as lighting. At all points care was taken to minimise bumping and other stressors.

Different colonies were labelled as they arrived with letters. Colony age and size was highly variable on arrival, with as few as five bees in some, with as many as 100 in others.

2.3.3 *D. melanogaster* Fruit Flies

D. melanogaster stocks were kept in a 12h/12h L/D cycle at 25°C while in growth or for experiments; separate emergency stocks were also kept at 18°C. A list of stocks and their origins are in Table 6. Other stocks are named as they are used.

Table 6 – Showing the stock names and Bloomington codes for each *D. melanogaster* wild type strain used

Strain	Source
OREGONR	Bloomington 25211
CantonS	Isaac Lab University of Leeds
Dahomey	Bretman Lab University of Leeds
W1118	Vienna 60000

2.4 Assays

2.4.1 Fruit Fly

2.4.1.1 Virgin Collection

Fly vials with eclosing pupae were emptied of adults at ~9pm in day prior to virgin collection, then placed overnight in an 18°C incubator. At 9-10am the day of virgin collection all adults were removed from tubes, anaesthetised on ice, and sexed via presence or absence of male genital pore under a binocular dissection microscope (Leica) with goose-neck lighting. The vials were then returned to 25°C. Within four hours the vials were retrieved from 25°C and all adults removed and anaesthetised and sexed as before, and vials returned to 25°C. This process was repeated until 9pm, or until enough virgins had been collected. Virgin females and virgin males were separated and allowed to recover at room temperature on cornmeal media, before being placed at 25°C for at least 24h before proceeding.

2.4.1.2 2-Day Assay

An assay vial was prepared by modifying a 50 ml falcon-style centrifuge by cutting the tip off and replacing it with a cotton wool bung. Two-three layers of filter paper (Whatman type 1) were placed in the cap. 500 µl of liquid food was placed on the filter paper, and 20 µl of either treatment or solvent was added. ~10 24h-old virgin female flies were anaesthetised and added to the tube. They were allowed to recover and then placed at 25°C 12h/12h day/night cycle for 48 hours. After which the flies were anaesthetised on ice and dissected.

2.4.2 Honeybee

2.4.2.1 Cage Assay

Some frames of nearly-eclosing capped brood were taken from hives the day prior to cage setup. All adult bees were removed and the frames were placed inside a dark incubator at 35°C. Within the next 24 hours all adult bees emerged from the frames were taken and mixed together in a bucket, then distributed into metal cages (10 cm x 10 cm x 5.5 cm steel with removable glass front and air holes, www.small-life.co.uk), at 80-120 bees per cage, depending on available newly-eclosed workers. The bees were supplied with food and water *ad libitum*, and treatment, re-applied daily. Generally, each assay contained at least one cage of QMP positive control (0.1 Qe /day), and an ethanol solvent control, both supplied as 20 µl aliquots onto a microscope slide placed in the bottom of the cage. 0.1 Qe was chosen as it has been shown to induce repression of honeybee worker ovaries *in cavea* in similar setups (104). 0.1Qe is a

higher concentration per bee than the workers would normally be exposed to in the hive, but is not so high that it stops producing repressive effect of worker ovaries (104). BP and EBO concentration and application is described in section 5.2.1.

After a number of days the bees were anaesthetised on ice and dissected.

2.4.3 Bumblebee

2.4.3.1 Cage Assay

Methodological development of cage assay is detailed in section 4.2.2

2.5 Dissections

All dissections were performed under binocular dissection microscopes with goose-neck lighting, usually applied laterally.

2.5.1 Honeybees and Bumblebees

Bees were anaesthetised by cold before all dissections. For ovaries and fat body, the abdomen was removed from the thorax. The dissections were carried out using high precision 55 forceps, under PBS. Different tissue was treated differently:

- For fat body, the fatbody was removed from the lining of the abdominal cavity.
- For brains, the head cavity was opened using a scalpel (Swann Morton 11), hypopharyngeal and mandibular glands removed before the entire brain was removed from the cranial cavity, retina and ocelli were stripped off.
- For ovaries, the entirety of both ovaries were extracted

Tissue was then processed according to need. For samples destined for RT-qPCR or HPLC, they were flash frozen in liquid nitrogen or on dry ice. For samples intended for morphology analysis, they were placed on a double depression slide in PBS.

When hundreds of bees were being processed for ovary extractions for morphology (i.e. not for molecular processing of tissue), it was common practice to process dozens of bees in one go. Splitting all bees from a given cage into their abdomens, and euthanising the heads and thoraces in soapy water, a maximum timescale of euthanasia to imaging was 90 minutes in order to prevent tissue degradation inhibiting Hess score characterisation.

2.5.2 Fruit Flies

All dissections were performed under iced PBS. Anaesthetised flies were placed in cold PBS and all ovaries extracted in batches. Flies were euthanised as they awoke by crushing thoraces as necessary. Ovaries were then stored in PBS on ice before processing.

2.6 Analysis

2.6.1 Honeybee

2.6.1.1 Ovary Morphology

Pictures were taken of each ovary at 25x magnification, and then analysed *post hoc* using the Blinder software (218). The ovaries were scored according to a modified Hess score: 3 = mature oocytes present; 2 = egg yolk visible; 1 = differentiation between oocyte and nurse cells visible; 0 = no visible differentiation of oocytes and nurse cells. These can be seen in Figure 2.1

Blinder also includes a quality control (QC), whereby random images are given more than once to allow for checking of consistency. All QC was >85% accuracy. If QC was lower than this, then the process was repeated at that moment, or on a different day. The QC results also provide a breakdown of which ovaries were inconsistently scored: if there were any differences in scoring of specific ovaries, they were double checked. Invariably, errors only occurred with labelling between ovary scores 0 and 1, which exhibit very subtle differences in phenotype.

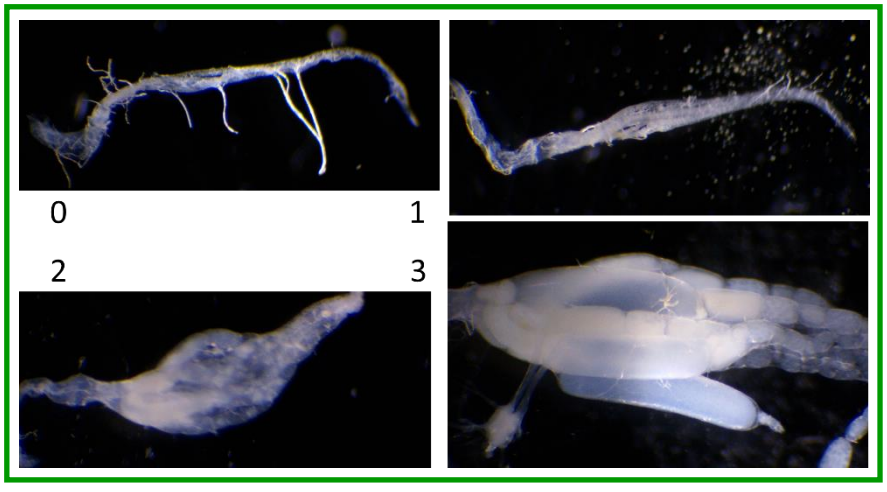


Figure 2.1 – Showing the four different stages of the modified Hess score 3 = mature oocytes present (bottom right); 2 = egg yolk visible (bottom left); 1 = differentiation between oocyte and nurse cells visible (top right); 0 = no visible differentiation of oocytes and nurse cells (top left).

The Hess score boundary of 1-2 was used throughout this thesis as the “inactive-active” boundary respectively, due to yolk deposition being an easily identifiable marker for definitive ovary activation. The similarity between 0 and 1 scored ovaries is not reliable enough to produce a predictable phenotype at the magnifications possible within our setup, even if it more accurately reflects the likely first indication of ovary activation from a developmental perspective. The 1-2 boundary therefore reflects a much clearer phenotype, akin to the active/inactive phenotypes used in the literature e.g. (46).

The raw Blinder data was exported and classified before import into R. The ordinal package was used to conduct a cumulative linked mixed model (CLMM) with post-hoc pairwise tests of the emmeans package using the linear predictor and score type. The CLMM was checked with a chi-squared test. Covariates, such as the day of experiment, or biological repeat, were checked for significant predictor effect. If they were not significant, they were adjusted to random-effect variables with the appropriate nesting for each variable.

The CLMM represents an ordinal mixed model, which while arguably inappropriate for the largely qualitative data represented by the Hess score, functions as a useful proxy. Although not truly ordinal, a Hess score does provide a linear set of developmental criteria that approximates ordinal data. It also allows for a more complex form of modelling, if imperfect, than can be produced from simply using inactive/active categorisation.

2.6.1.2 Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR)

For evaluating gene expression in honeybee tissue, samples were flash frozen on dry ice or in liquid nitrogen and stored at -80°C until used.

Primers were first designed by obtaining the sequence of the target gene from the NCBI genome database, using the mRNA and gDNA sequence to align the gene using Splign (219) to identify introns and exons. Primers were designed where possible targeting the intron exon boundaries in order to produce mRNA specific target products. Primers were then designed using Primer3 (220) selecting for primers with products of ~100bp, 50% GC content, differing in Tm by at most 1°C, at roughly 60°C and with pair and cross dimerization with ΔG less than 4 using Beacon Designer (Premier Biosoft). The generated primers were then blasted against the *A. mellifera* genome on NCBI to identify any cross-targets. Primers were synthesised by Integrated DNA technologies. Tissue was homogenised in Tri-reagent (Zymo), by mechanical pestle for 60 seconds, before being passed through a 25-gauge 1 ml hypodermic needle 10 times. RNA was extracted using Direct-zol RNA microprep kit (Zymo), then quantified using a nanodrop.

Samples were given an additional separate DNAse treatment using the ds DNAse (ThermoFisher Scientific) kit, followed by the reverse transcription using the RevertAid ThermoFisher kit. After cDNA synthesis the samples stored at -80°C.

The samples were later thawed and used for qPCR. qPCR was carried out using SYBR green on a BioRad CFX connect machine using BioRad CFX maestro; using ~1 ng of cDNA per sample and 450 nM primer, with each sample run in technical duplicate, or triplicate for primer efficiencies. Analysis of expression was done using gene study function on BioRad CFX maestro.

Gene expression values were then exported into R, where the distributions of expression for the given treatments was ascertained by Cullen and Fray and diagnostic graphs, followed by a GLMM using covariates of confounding variables. If the distribution was Gaussian, an F-test was used to determine the predictor effect of each variable, while a Chi-squared was used for gamma and poisson distributions. Covariates were then removed if statistically non-significant and adjust to random-effect variables with the appropriate nesting. Diagnostic plots were also used to assess the fit of the model distribution. *Post hoc* pairwise tests were carried out in the emmeans package.

2.6.1.3 Kaplan Meier Survival Curves

Kaplan Meier survival curves were generated using the survival and survminer packages in R. A Surv object was generated from mortality data and fit using the survfit function relative to the given variable of investigation (Food type or Treatment type etc.). This object was then used to generate a cox mixed effect model with cage nested in treatment. This was then processed for post-hoc pairwise Tukey testing via the emmeans package. The model was tested for predictor effect against a null model using a Log Ranked Test (LRT), and chi-squared test.

2.6.2 Fruit Fly

2.6.2.1 Ovary Fixing

After extraction, ovaries were fixed in 1.5% formaldehyde for 10 minutes at room temperature (~20°C). The ovaries were then washed in PTx (PBS with 0.1% TritonX 100), by removing the supernatant and replacing with 1ml of PTx. The ovaries were then incubated in 1ml of PTx for 60 minutes on a rotator. If the ovaries were to be stained with DAPI (4’,6-diamidino-2-phenylindole), then the PTx was replaced with fresh PTx and 1µl of DAPI was added, mixed by inversion then incubated on a rotator in the dark for 10 minutes. The ovaries were then washed twice more. Whether DAPI was added or not, the ovaries were then stored in 70% glycerol (diluted into PBS).

2.6.2.2 Ovary analysis

The ovaries, having been left in 70% glycerol for at least 8 hours (overnight), were bridge mounted onto a microscope slide (roughly 9 ovaries per slide). The ovaries were teased apart with probes, and then the top cover slip added and compressed to spread out the ovaries for egg counting. Each stage 14 vitellogenic oocyte (221) per ovary was counted under a binocular dissection microscope with lateral lighting.

The number of stage 14 eggs per ovary was categorised according to sample and imported into R, where, using the fitdistrplus package, the distribution was ascertained by using a Cullen and Fray graph using the descdist function followed by running data compared to model discrete distributions in the denscomp, qqcomp, cdfcomp, and ppcomp functions. Once the most appropriate distribution was found, a GLMM was constructed with repeat and day processed as random effect variables. Once the distribution was ascertained, if the distribution was normal, then an F-test was carried out to investigate the F-factor, degrees of freedom, and to check for the appropriateness of variable selection; if the distribution was not normal, then a chi squared test was done to investigate the same statistical values.

Post hoc pairwise tests were carried out in the emmeans package.

Chapter 3 How does *Apis mellifera* respond to QMP?

3.1 Introduction

The Western honeybee *A. mellifera* is an organism which has been of an intrinsically high cultural and economic value in society since the development of human civilisation (222).

The eusocial system of organisation that honeybees possess demonstrates a level of social complexity rarely matched in the animal kingdom. This high degree of sociality is an incredibly successful ecological strategy, as honeybees are able to aggressively outcompete any non-eusocial species in their ecological range (223).

The reproductive division of labour exhibited in this animal demonstrates Hamilton's law of indirect fitness taken *ut extremis* (60), with a single reproducing queen providing the indirect fitness of as many 65,000 workers for an average hive in the summer peak (224).

3.1.1 QMP-mediation of the reproductive division of labour

The reproductive division of labour so essential to this eusocial system in the honeybee, is mediated by many redundant pheromones and pathways (47), but the most well studied, and likely most important of these pheromones is QMP (47, 102, 214). This complex mixture results in the repression of reproduction in workers, as well as many other characteristics of social organisation.

The proposed model for QMP activity, detailed in section 1.3.1.1, is that QMP binds to an odorant or gustatory receptor in the antennae/mouth/gut/legs. This signal is transduced to the brain where endocrine, or neuroendocrine, signals travel through the haemolymph to the ovary, possibly via the fatbody, where ovaries are then repressed in the germarium (103).

Although this model is reasonable, there are potential problems with the assumption of the ordering of these pathways. It is known that physical touch is required for QMP-mediated response (111); it is known that hormone levels change with QMP exposure (113, 114); and that dopamine level changes in the haemolymph in response to QMP indicate somatic signalling of some kind (104). However, the ordering of these signals is not known. A possible alternative explanation is that QMP acts directly on the ovary or fatbody via diffusion from the gut, and that the systemic changes observed are as a result of the direct influence of QMP on these tissues signalling towards the brain and via the haemolymph.

The role of QMP in non-repressive roles obfuscates the mechanism by which it might work in the ovary. It is possible that the odorant receptor known to be responsible for the sex

pheromone aspect of QMP activity in drones, OR11 (110) is active in workers and aids in the signalling of QMP internally within the bee, but it might not be related at all. Additionally, the queen-retinue-response, whereby the workers retinue to the queen and antennate and lick her body occurs in response to the presence of QMP (108). These behaviours strongly imply that the workers are responsive to QMP at a releaser level, via the antennae. But it is not clear whether these are the mechanisms through which repression is brought about.

The role of Notch signalling in the QMP response also does not elucidate this difference (132), as the Notch signalling indicates a quasi-paracrine tissue response, it does not indicate mechanism of signalling upstream of this activity. It could very well be that Notch signalling occurs in response to QMP ovary inhibition in this tissue.

As described in section 1.3.1.1.5, Ronai et al. (140) suggested that PMP34 is the genetic basis of queen control in honeybees. However this seems unlikely, due to the methodological issues discussed previously.(140, 143, 144)It has also been shown that QMP does not inhibit the ovary activation of queens, and that this is possibly due to a U-shaped response curve to this pheromone. I.e. that QMP only inhibits ovary activity at the concentrations a worker would be exposed to in the hive, and when exposed to higher concentrations (e.g. at the level the queen exposes herself to) workers reproduction is no longer suppressed (104).The pheromone-mediated repression of ovary activation in workers, but not queens, is a form of adult plasticity response, similar to diapause.

3.1.2 Plasticity and Diapause

As has been discussed in section 1.4.2.1, honeybees possess a form of diapause in which, during winter conditions the queen deactivates her reproduction. This plasticity in queen ovary activation and repression, may be reflective of a similar plasticity in workers.

It has been shown that workers have the ability to deactivate their ovaries during the process of activation by the presence of a queen (I.e. that workers in the process of activating their ovaries due to isolation from queen or QMP, if returned to an *in alvo* context, revert to being reproductively repressed) (225). In this paper a small number of 30 callow worker honeybees were assigned to cages containing comb, along with sugar water and pollen cake *ad libitum*. After two weeks, 33-66% of the bees were dissected to determine ovary activation, and the remainder were sprayed with sugar water (to deter aggression), and placed into queenless and queenright host colonies (ordinary hives with brood present). After one additional week, all bees, both experimental and host colony, were flash frozen and dissected according to similar

methodologies used in this thesis. An additional control of workers kept in the starting conditions for three weeks.

This paper has certain methodological issues discussed later in section 3.4.1.1, however it raises questions as to the limits of this plasticity. It is known, for example, that foragers are not receptive to QMP via retinue response (226). Foragers have no reason to provide retinue behaviours to the queen, as they spend much of their time outside the hive and this time they spend away does not result in the activation of their ovaries (227).

Consistent with the reproductive ground plan hypothesis, the uncoupling of behaviours relating to reproduction and foraging is likely a precondition for the evolution of eusociality (66). This perhaps also holds true for the plasticity which workers are able to exhibit in relation to their ovary activation.

In the fruit fly, reproductive diapause is an incredibly plastic response, inducible via cold/heat, chemical, or food stress. In this state, many aspects of the behaviour and biology of the fly change, but of particular interest is the suppression of egg-laying (199). Indeed, in diapause in this organism the ovaries regress, becoming much smaller, and with far fewer developed eggs present.

A similar phenotype is visible in the honeybee. The winter phenotype observed in this species, sees the queen likewise show a significantly reduced ovary size (187), as well as many of the other behavioural changes which occur in the fruit fly (such as changes to physical activity). There are also significant differences between these diapause phenotypes however, possibly due to the fact that, although the individual honeybee is exothermic like all insects, the honeybee colony very strictly regulates its temperature. As a result, much of the physiological activity of this species does not change so as to allow continued temperature regulation of the hives, which maintains at least 35°C (228), even when external temperatures are as low as -20°C (52).

The reduced ovary activity seen in queens also manifests in the workers, which stop activating their ovaries once the winter bee phenotype has begun (229).

3.1.2.1 Toxicity and Feeding Effects of QMP and Diapause

Given that QMP component HVA is known to repress negative memory learning in honeybee workers (by limiting the ability of workers to associatively learn in response to mild shock stimuli via olfactory processes) (230), it has been suggested that the queen retinue response is used to inhibit some negative element of QMP exposure, as this is thought to be an important part of the mechanism by which QMP is dispersed throughout the hive (231). Despite this,

there is no confirmation of whether there exists any toxic effects of QMP on honeybee workers, and whether these toxic effects explain the reproductive repression seen.

Similarly, there is evidence in fruit flies that QMP induces a starvation-like response (183), and there is similarly no confirmation of this response in honeybees either.

The inducing of diapause response via chemical toxicity or starvation is well understood in fruit flies (183, 232). However, the role of toxicity and starvation on the reproductive division of labour in honeybees is very poorly studied.

3.1.3 Evolution of eusociality in the honeybee

Understanding the complexity of QMP via exploring the mechanisms of its action is particularly relevant to understanding the evolution of this species, as other hymenopteran eusocial insects do not seem to exhibit the same complexity of queen pheromone has honeybees do (discussed in more detail in section 1.3.1.1.6). Understanding the evolutionary pathway that has resulted in the complexity of QMP via the elucidation of its mechanisms, also gives us general insight into the evolution of eusociality as a whole within the hymenoptera, and possibly helps to settle the debate that occurs around the nature of queen pheromones evolving as honest signals of fertility of the queen, or as forcible reproductive constraint of the workers by the queen.

3.1.4 Research Questions

The research herein will attempt to answer the question, what are the mechanisms by which QMP is able to bring about repression of reproduction in honeybee workers?:

- What is the outcome of QMP exposure to honeybee workers on feeding, and toxicity? And how does starvation affect QMP-mediated reproductive repression?
- What are the limits of honeybee worker plasticity? And how does this compare to queen-diapause responses?
- Are the known internal hormonal signalling changes a result of QMP signalling via the proposed model? And if so, what is the sequence and nature of that signalling?
    - Does QMP exposure result in observable changes in expression of signalling pathways in different tissues in a sequential manner? As predicted by the model
    - How do these signalling pathways relate to known existing mechanisms?
- What do these data tells us about the evolution of queen pheromones in this species? And about the nature of the evolution of eusociality in the honeybees?

3.2 Chapter Methods

3.2.1 Honeybee caged experiments

All *in cavea* honeybee experiments in this section, except for the experiment detailed in section 3.3.1 are fed on the FandP diet (see section 2.2.3.1.2).

3.2.1.1 Food type effects

Cages were set up as detailed in section 2.4.2.1, with different feeding regimen.

For each repeat, three feeding methods were used, with QMP+/- for each feeding method. The feeding types are laid out in Table 7.

Table 7 – Showing the proportion of each food type given for food-type effects on *A. mellifera* worker ovary activation

Feeding type	Pollen per day /g	Fondant per day /g
FandP	1.5	1.5
Pollen only	3	0
Fondant only	0	3

Bees were dissected and ovaries imaged for analysis at day 10 of the experiment.

3.2.1.2 Adult worker ovary plasticity and QMP-period experiments

Cages were set up as in 2.4.2.1.

Each repeat contained a QMP+/- control in their own incubators and a treatment. The treatment group was shifted between incubators as appropriate:

1. For investigating the ability of QMP to repress already-activated ovaries, the treatment cage was kept in ethanol control treatment conditions for 10 days, before being exposed to QMP for a further 10. The QMP+/- controls were kept in their respective treatment conditions for 20 days (detailed in Figure 3.7).
2. For investigating the start of the QMP period, the treatment cages were kept in ethanol control treatment conditions for three or five days before QMP was introduced for the remainder of the 10 days. The QMP+/- controls were kept in their respective treatment conditions for 10 days (detailed in Figure 3.16).

3. For investigating the end of the QMP period, the treatment cages were kept in QMP+ conditions for six, eight, or 10 days, and then QMP was withdrawn for a subsequent 10 days in all cases. The QMP+/- controls therefore were kept in their respective treatments for 16, 18, or 20 days depending on the experiment (detailed in Figure 3.19).

At the end of the experiment all bees were dissected: ovaries were extracted and imaged.

3.2.1.3 *In alvo* worker ovary activation

In order to age match the bees, freshly eclosing bees were marked with non-toxic paint on the top of their thorax (BOSCO paint pens), and then released into a queenless hive. Different colours were used for different days so as to differentiate during recollections.

After 28 days (to 30 days), bees were identified by colour and placed into cages. These cages then formed the treatment and ethanol solvent control groups of a cage assay, with the QMP+ control being freshly eclosed adults.

In total the recovery rate of bees from the queenless hive was about 10% of marked bees. With the remainder either having died, or much more commonly having laterally migrated into adjacent hives. Marked bees were found in every single hive of the 12 in the apiary, despite having been manually returned only to the queenless hive. This potentially represents a significant sampling bias, but it was chosen not to retrieve coloured workers from adjacent queenright colonies due to their exposure to the repressive pheromones present in those colonies.

Cage assays then progressed as in general methods, with the treatment cage being exposed to QMP for 10 days before dissection.

At the end of the experiment, all ovaries were extracted and imaged.

3.2.1.4 Investigating gene expression timings for single ovaries

Cages were setup as per 2.4.2.1 for QMP+/- treatment groups. On each of days zero, two, four, six, eight, and 10, five bees were dissected for their ovaries, brains, and fat bodies from each cage, and flash frozen at -80°C for RT-qPCR analysis. Ovary scores were ranked according to modified Hess score and marked on each tube. At day 10 all remaining bees were dissected for their ovaries and imaged.

RT-qPCR was carried out as in section 2.6.1.2. The primers used in this experiment are detailed in Table 16. For several of the genes investigate, such as *bHLH2*, and *her*, as well as for several of the reference genes, such as *rpn2* and *mrpl44*, the PCR plates repeated on different days

using the same template in order to confirm that the individual variation seen wasn't the result of technical error. The results of these technical repeats was that there was no difference between plates and so the data has been omitted (but is available upon request from the Duncan Lab at the University of Leeds).

Various genes were investigated during this experiment, many of which were unnamed in the genome characterisations, so names were assigned. These are laid out in Table 8.

Table 8 – Showing the gene names used in this thesis, and their respective genome references

Gene	Genome Reference
DDI1	LOC552049
Lily	LOC410857
Puff	LOC408565_1
Red	LOC411392
Shell	LOC408763
Trev	LOC726875
Unk1	LOC725233_1
Zinc1	LOC410222_1
Zinc2	LOC410108

3.2.1.5 Investigating the potential for precocious forager development

Honeybee cages were set up as per 2.4.2.1 with QMP+/- treatment groups. At day 10, ovaries were extracted from all bees and imaged to check for QMP-mediated repression. From each treatment, honeybee ovaries were extracted and scored as either inactive (Hess 0-1), or active (Hess 2-3). For six each of active and inactive ovaries per cage, brains were also extracted and flash frozen at -80°C for RT-qPCR. Resulting in six brains from each treatment QMP+/- and reproductively active/inactive bees.

In addition to these samples, six controls of nurses, guards, and foragers were taken from the hives in the apiary. Foragers were identified by the presence of pollen in their pollen baskets. Nurses were identified by the observation of workers attending to larvae-filled cells. Guards were identified by presence in entrance of hive, shaking of abdomen in Nasonov pheromone exhibition, and "checking" behaviour of incoming workers. Brains were extracted and flash frozen at -80°C.

RT-qPCR was carried out as in section 2.6.1.2, using the primers detailed in Table 15.

3.2.2 Statistics

3.2.2.1 Global feeding analysis

Graphs were produced in R using the ggplot2 package and finished in Microsoft PowerPoint. Means of each average consumption for each day were calculated and standard deviation used for error bars. For analysis, the difference of food given to the bees and food removed from the bees 24h later for each cage was calculated into a feeding difference value for each of fondant and pollen. This value was then used to do individual pairwise comparisons between each of the treatments for each day via GLM using a distribution calculated via the descdist package. For gaussian fitted models, an ANOVA was performed using an F test, while for the gamma fitted models, a Log Ranked test was used to generate significance values for a given time point.

Overall significance of treatment effect on food consumption was also calculated using the data aggregated across all days, using a GLM with gaussian distribution. Cage was initially introduced as a covariate, but was found not to significantly predict consumption difference, and so was excluded. Distribution was determined using the Descdist package. Significance was determined using ANOVA with F-test.

3.2.2.2 Reference gene analysis

All reference gene analysis was undertaken using the raw Cq values and GeNorm2 function of the ctrlgene package in R to generate M-score. The V-score was then generated using the pairwiseV function. Graphs were plotted using ggplot2 and adjusted in Microsoft PowerPoint.

3.2.2.3 RT-qPCR analysis

Graphs were produced using ggplot2, and finished using Microsoft PowerPoint. Analysis was performed via glm with gaussian distribution, and post-hoc Tukey pairwise tests.

3.3 Results

Despite *A. mellifera* being a species of high cultural and economic importance (233), the internal mechanisms by which the social structure of this species are maintained are poorly understood.

The reproductive division of labour in honeybee colonies, essential for its eusociality, is maintained by the presence of queen pheromones (particularly QMP) inhibiting the reproductive potential of workers. (234)

There is a lack of understanding of the mechanisms by which QMP is able to bring about this reproductive constraint. How the known hormones and signalling pathways are acting and in which order; the limits of worker plasticity in response to honeybee QMP; the relationship of this plasticity to known diapause responses; and the role of toxicity and starvation in the mechanism of QMP in this species.

3.3.1 Fondant and Pollen diet does not interfere with QMP-mediated repression in *A. mellifera* workers, and increases survival *in cavea*

First, in order to investigate the mechanism of action of QMP on ovary development in worker honeybees, methodological problems had to be addressed.

The previous work conducted and published by the Duncan lab used CBF as a sole food source for *in cavea* experiments (e.g. Duncan et al. 2016 (132)). This food source was used as it is particularly high in protein, thus maximising the possibility of an activated ovary phenotype in accordance with the published data on this subject, which shows that increased protein content results in increased ovary activation (235). In this scheme, if QMP were able to repress reproduction of this phenotype it would demonstrate that it is biologically significant.

However, CBF appears to result in relatively high mortality in cages. The 10-day experimental time was chosen, as beyond this mortality is too high to continue experiments (104, 123). The bees, which ordinarily primarily consume carbohydrates in the form of honey as adults, were probably overconsuming the CBF in order to meet their carbohydrate intake. This, combined with the fact that bees do not defecate *in cavea* due to the naturally high degree of cleanliness shown by honeybees *in alvo*, resulted in large distended guts, filled with CBF, which were particularly fragile and prone to bursting

By changing the food source to a more biologically relevant sugar and protein diet (coined FandP, for fondant and pollen), whereby both food sources are given and replaced daily to allow a choice between foods, the bees would gorge less, and so live longer in cages, allowing

for longer experiments to be undertaken. It would also theoretically lower mortality rates by day 10.

The first step was to assess whether the change in food brought about a change in responsiveness of the workers to QMP.

As can be seen in Figure 3.1 (χ^2=53.642, df=3, P<0.001) both CBF and FandP were able to produce QMP-mediated repression of worker ovaries. There was also a significant difference in activation between CBF and FandP fed bees not exposed to QMP, consistent with the published data that higher protein diets result in greater ovary activation. Notably there was no significant difference between QMP+ samples, highlighting the consistency of the repressed phenotype across feeding regimens. (individual pairwise comparisons in Table 17, in appendices)

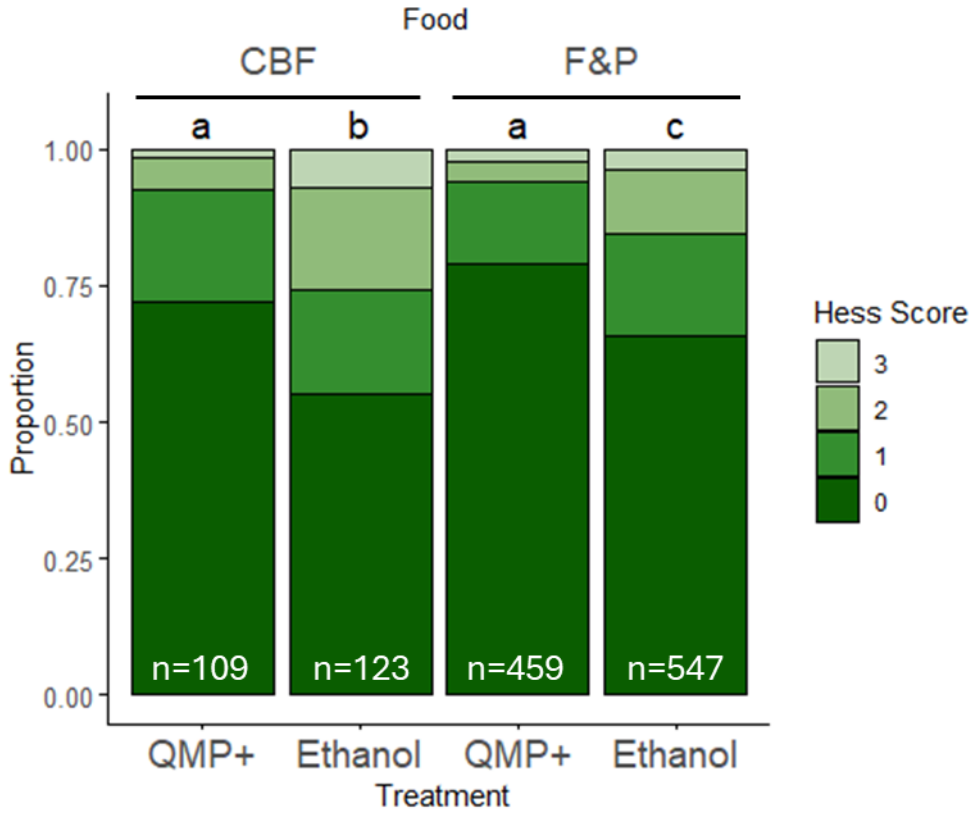


Figure 3.1 – Showing a stack proportion bar chart of ovary activity of *A. mellifera* workers fed under different feeding regimens and exposed to QMP. The y-axis shows the proportion of ovaries of a given Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present), while the x-axis shows treatment conditions. The two bars on the left show bees fed on CBF and the two bars on the right bees fed FandP. Significance is given as letters (p<0.05).

The mortality results in Figure 3.2 show a strongly significant difference in mortality between diets (χ^2=2835, df=1, P<0.001), with much higher survival probability for the FandP diet consistent with previous *ad hoc* observations.

In addition to these data, anecdotally it was also observed that the abdomen of workers was less distended by day 10, and during dissections that the gut was far less filled.

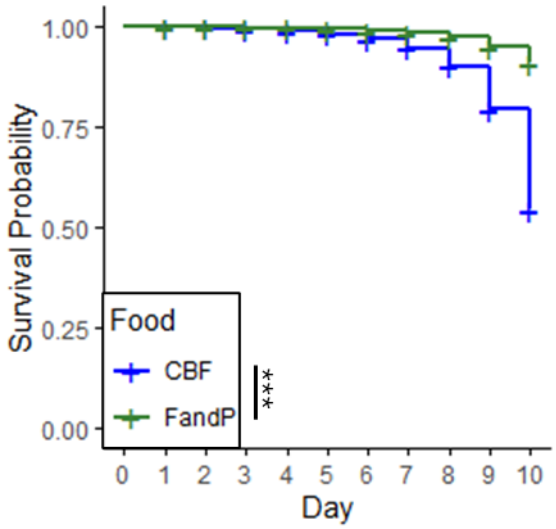


Figure 3.2 – Showing a Kaplan-Meier survival curve of the different feeding regimens on *A. mellifera* worker survival. The y-axis shows survival probability, and the x-axis shows time of experiment in days, the different colours show different feeding regimens. Significance given as ***=P<0.001, and calculated via Cox mixed effect model and post-hoc chi squared test. There is a significant difference in survival probability between the two feeding regimens.

3.3.2 Aggregated Feeding Data

Throughout the course of 2023 and 2024, every *in cavea* honeybee experiment undertaken included QMP+/- internal controls, generating a large dataset from which macro-observations regarding the nature of QMP can be drawn.

Mortality and food consumption were recorded every day for 152 cages, of which 109 were either 0.1Qe QMP per day, or ethanol solvent control.

3.3.2.1 QMP exposure increases mortality in *A. mellifera* workers *in cavea*

It has been observed in *D. melanogaster* that QMP exposure results in an increase in mortality for females (but not males; Duncan lab personal communication). Figure 3.3 shows that *A.*

mellifera workers also show this effect, whereby there is an effect of increased mortality in QMP-exposed workers (χ^2=7023.5, df=1, P<0.001), though with small effect size. The increased mortality is possibly also indicative of sub-lethal toxicity effects of QMP, as ordinarily decreased reproduction leads to increased longevity (236), although the longevity-fecundity trade-off (the observation that as animals, and particularly insects, reproduce more they shorten their lifespan (236)) is known to be reversed in eusocial insects (237, 238).

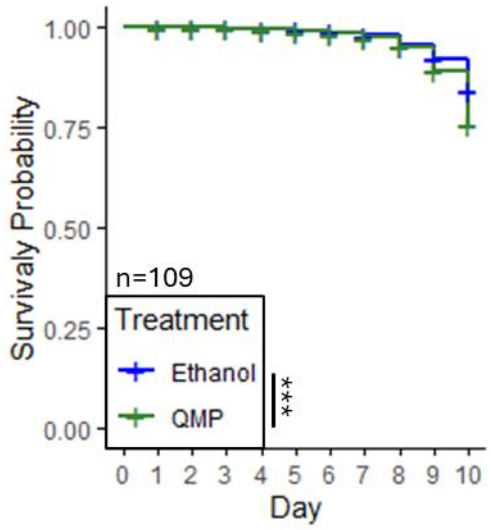


Figure 3.3 – Showing a Kaplan-Meier survival curve of *A.mellifera* workers exposed to QMP or solvent control. The y-axis shows survival probability, and the x-axis shows time of experiment in days. Treatment is shown via colour. Significance is shown as ***=P<0.001, calculated via cox mixed effect model and post-hoc chi squared test. The sample size was aggregated from the controls of various experiments, with the n-value equalling the number of cages analysed. There is a significant effect of QMP on survival.

3.3.2.2 QMP exposure of *A. mellifera* workers *in cavea* does not change protein consumption, but does increase carbohydrate consumption

It is generally known that increased protein intake is necessary for organismal growth, and this is especially true of egg-laying insects, which require increased protein for optimal egg-laying rates (239). As a result, we should expect to see the solvent-control exposed bees, who generally show greater ovary activation, to consume greater amounts of pollen, which is mostly protein.

It has also been observed in *D. melanogaster* that QMP induces a starvation-like response (183). If the mechanism of action of QMP-mediated repression were similar between fruit flies

and honeybees, we should also expect to see a similar observation here, and see QMP exposed bees consume greater amounts of fondant, which is mostly carbohydrate.

As can be seen in Figure 3.4**A** and **B**, in both years there was consistently no difference in pollen consumption between QMP and solvent exposed bees (**A**: F=0.0067, df=653, P=0.935; **B**: F=0.685, df=660, P=0.408). This disproves the original hypothesis that pollen consumption would be different between treatments on account of the need for protein supply as a precursor to egg-production.

In this figure we do also see an increase in fondant consumption for bees exposed to QMP (**A**:F=11.873, df=653, P<0.001; **B**: F=14.462, df=660, P<0.001), consistent with the proposed hypothesis that QMP induces a starvation-like response, as in *D. melanogaster*. (115, 230, 240, 241)

The difference in fondant consumption between treatments is not consistent across all 10 days however, with less consumed at the beginning and at the end of the 10 days. There is much higher fondant consumption in days 5-7 for both years, as a measure of effect size, however 2024 appears to have more consistently higher fondant consumption between days than in 2023. (The results of pairwise test comparisons can be seen in Table 18 and Table 19 for 5**A** and **B** respectively, and the results of effect size are in Table 20 and Table 21 for 5**A** and **B** respectively, in appendices)

Additionally, and perhaps most markedly, we see a behavioural switch occur throughout these first 10 days. Initially the bees only consume pollen and not fondant, and by day 10, this has inverted to no pollen consumption and high fondant consumption. The switchover happens in both years between day four and day five of the experiment. The initially high pollen consumption is consistent with that observed previously (235, 242)

The switch occurring is perhaps because development in honeybees does not end with eclosure from pupae, and that *in alvo* the extra protein needs are linked to hypo-pharyngeal gland activation (for the provision of royal jelly, as is a task for nurse bees), or the activation of wax glands, and that these two tasks are not carried out *in cavea* due to the lack of brood or surfaces for adequate comb production (243). It is also possible that the sexual development of the workers is delayed due to the physiological needs of the queen, who delays activation of ovaries until after the mating flights, between six and eight days after eclosing from her pupa. QMP may have evolved to take advantage of this in delayed development in workers and represses this development as a form of reproductive constraint for the maintenance of the eusocial structure.

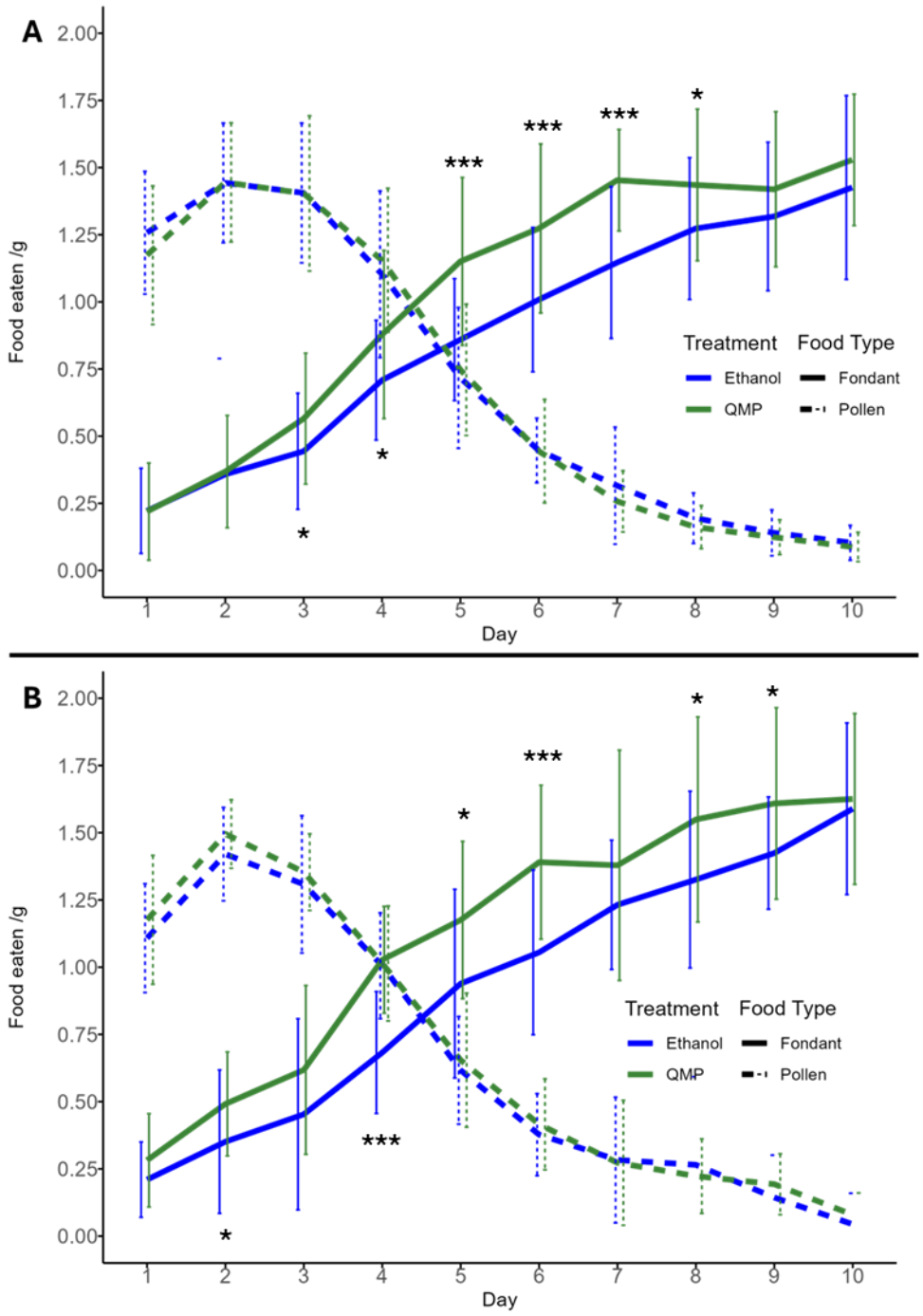


Figure 3.4 – Showing a line chart of the feeding data across all FandP fed control cages of experiments on *A. mellifera* workers in A**: 2023 and** B**: 2024.** The y-axis shows food consumed in grams, the x-axis shows day of experiment, colour denoted treatment of either 0.1Qe QMP or ethanol solvent control, dashed lines show pollen consumption and full lines show fondant consumption. The error bars are one standard deviation from the mean. Significances are given as *=P<0.05, ***=P<0.001, calculated via GLM and post-hoc F test (if model was fitted as gaussian), or Log Rank Test (if model was fitted as gamma).

Overall, four conclusions can be drawn about *in cavea A. mellifera* worker food consumption with regards to treatment from these data:

- QMP-exposure does not influence pollen consumption relative to ethanol solvent control.
- QMP-exposure results in an increase in fondant consumption relative to ethanol solvent control.
- This QMP-mediated difference in fondant consumption is not consistent over time and peaks in effect size at the day 4-7 range.
- There is a behavioural switch, independent of treatment, whereby bees initially consumer pollen, but switch over to fondant after a few days.

3.3.3 *A. mellifera* workers only fed fondant do not activate their ovaries, while those fed only pollen do not survive

It has been shown that high/low pollen diets are able to influence worker ovary activation rates (229).

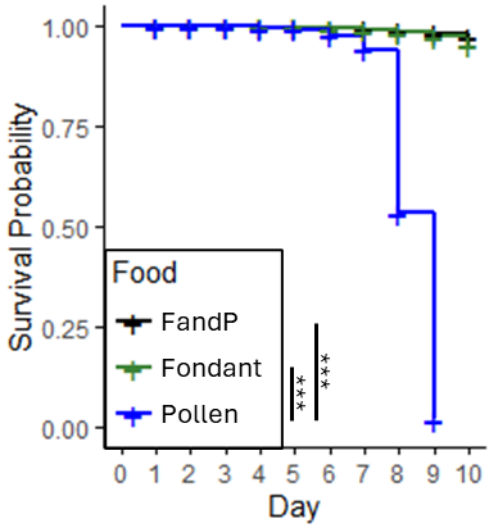


Figure 3.5 – Showing a Kaplan-Meier survival curve of honeybee workers which have been fed different food sources. The y-axis shows survival probability and the x-axis day of experiment, food is given as colour, where "fondant" is fondant only, "pollen" is pollen only and "FandP" is a choice of either. Bees fed only pollen could not survive to the end of the experiment, but there is not difference in survival between the other two food types.

However, the fact that workers eat pollen regardless of QMP exposure, perhaps implies that food consumption is less relevant to ovary activation, and therefore seems to provide counter-

evidence to the results of section 3.3.1, whereby there was an increase in ovary activity of QMP- control bees in CBF feeding conditions compared to FandP.

In order to help elucidate the relationship between food type and ovary activity, an experiment was designed in which the bees were fed FandP, pollen only, or fondant only, in QMP+/- conditions.

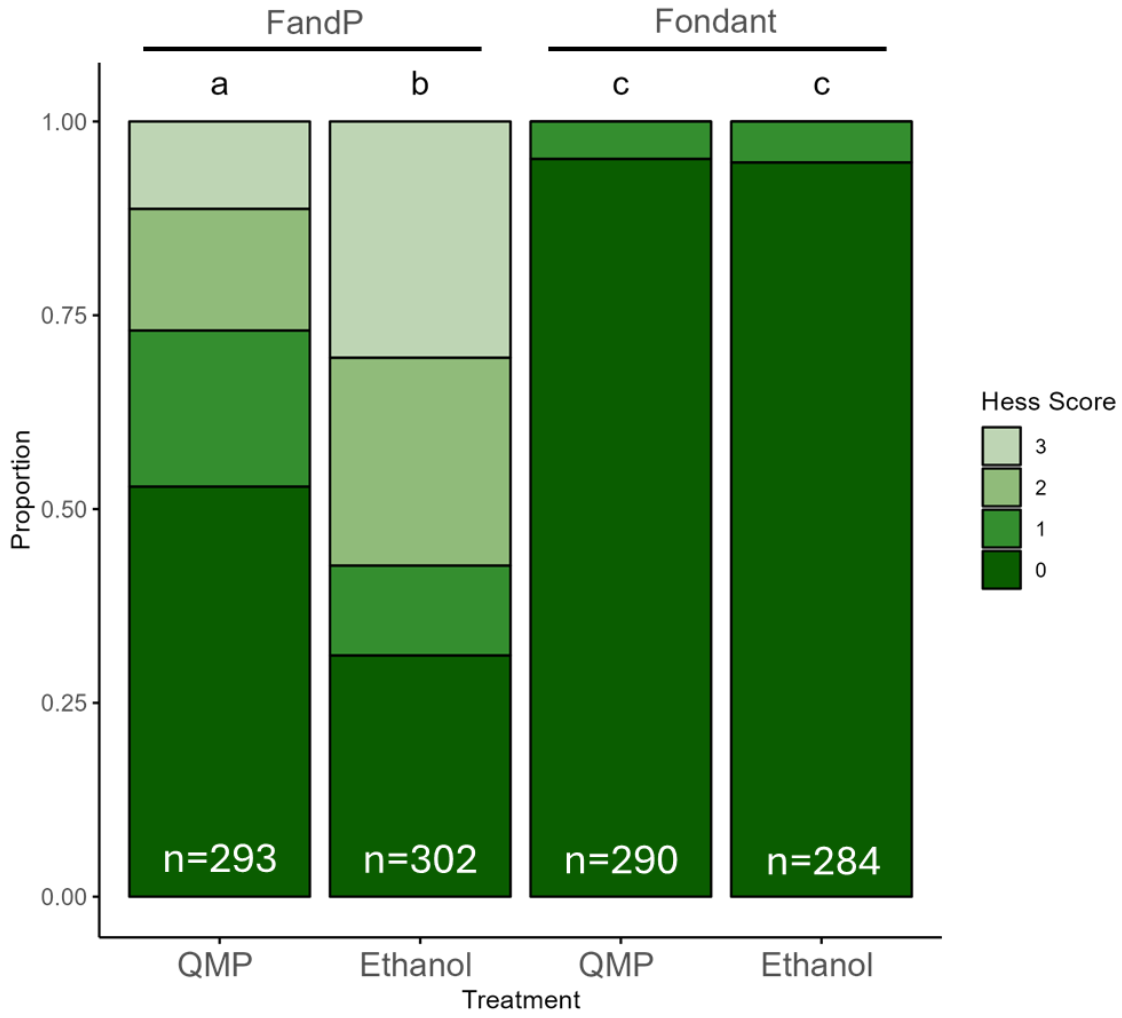


Figure 3.6 – Showing a proportional stacked bar chart of ovary activity of *A. mellifera* workers when fed different quality foods. Ovary activity was measured via modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present). The y-axis shows proportion of ovaries of a given Hess score, while the x axis shows treatment of either 0.1Qe QMP per day or ethanol solvent control, or food types of either FandP diet, or fondant only. Significance given as letters ($P<0.05$) calculated via CLMM and post-hoc Tukey pairwise test. Under the FandP diet, QMP is able to repress the activity seen in the ethanol control, but under fondant diet, no activation of ovaries occurs.

As can be seen in Figure 3.5, bees only fed pollen had no chance of surviving to the end of the experiment, showing that honeybee workers are unable to survive on pollen alone, but there was no difference in survival between the other two food types (results of individual pairwise tests in Table 26, in appendices).

Due to the 0% survival rate of the pollen-fed samples, only the other two feeding regimens were analysed for ovary activity, and these can be seen in Figure 3.6 (χ^2=309.97, df=3, P<0.001). As can be seen quite clearly from this figure, the *A. mellifera* workers only fed fondant showed zero degree of ovary activity (i.e. that not a single ovary exhibited the "activated" phenotype represented by a modified Hess score of 2-3) (results of pairwise tests in Table 34, in appendices).

The ovary scoring data in Figure 3.6, when compared to the data shown in Figure 3.4 (overall feeding data), show that the ovaries of workers not given any protein source do not activate, but in a different way to that of ovaries of workers fed QMP. I.e. that pollen is eaten regardless of eventual ovary activation or treatment type ,and this does not necessarily result in ovary activation (i.e. that pollen consumption is not a predictor of ovary activity, even though being forced to consume unnaturally high protein food results in greater activation), but removing pollen completely prevents ovary activation.

These data also confirm previous observations that pollen presence is necessary for ovary activation (244).

3.3.4 Plasticity of ovary development in honeybees

Most papers investigating the effects of QMP, or queen substance, on ovary activity in *A. mellifera* workers, do so by investigating the repressive effect of pheromone exposure on worker ovary development. I.e. by exposing young workers with inactive ovaries to pheromone in order to inhibit sexual development (132).

Only one group has shown that already-activated ovaries are able to be suppressed by exposure to queens (225). Traditional apicultural practice has it that once a colony has become queenless, there is a relatively short period in which a new queen must be produced by the colony before the colony no longer accepts a new queen, termed "hopelessly queenless" (245).

In order to reconcile the ideas presented in this paper and traditional understanding, the ability for QMP to repress already activated ovaries *in cavea* was investigated.

3.3.4.1 QMP exposure cannot induce ovary repression *in cavea* once ovaries have activated

First, in order to show whether QMP was able to repress ovary activity once activated, *A. mellifera* workers were allowed to activate their ovaries for 10 days (when a small subset of bees were dissected to check for expected activity) before being exposed to QMP for a further 10 days in an attempt to repress them, then dissected at day 20 (detailed in Figure 3.7)

The results of this experiment are shown in Figure 3.8 (χ^2=20.038, df=2, P<0.001), where the bees that had been allowed to activate their ovaries and then exposed to QMP showed identical phenotype to bees never exposed to QMP (results of pairwise tests in Table 22, in appendices). This shows that once activated, honeybee worker ovaries cannot be suppressed *in cavea* via QMP exposure.

There was no effect of survival probability between these treatments, as can be seen in Figure 3.9 (results of individual pairwise tests in Table 23, in appendices).

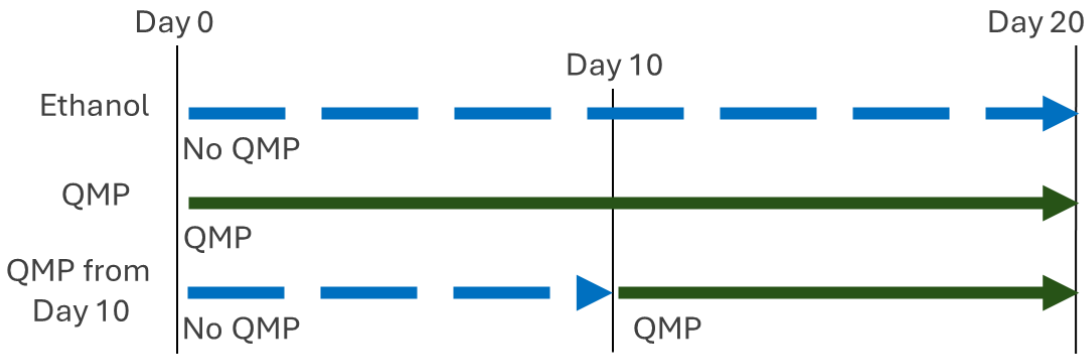


Figure 3.7 – Showing the scheme of the experiment investigating the ability of QMP to switch off active *A. mellifera* worker ovaries. Cages with bees exposed to ethanol are shown with dashed blue lines. Cages with bees exposed to 0.1Qe QMP per day are shown with solid green lines. The "QMP from Day 10" cages were initially exposed to ethanol control for 10 days, before being exposed to QMP for 10 more days. A subset of bees was taken at day 10 to check for activation and repression before the treatments were switched.

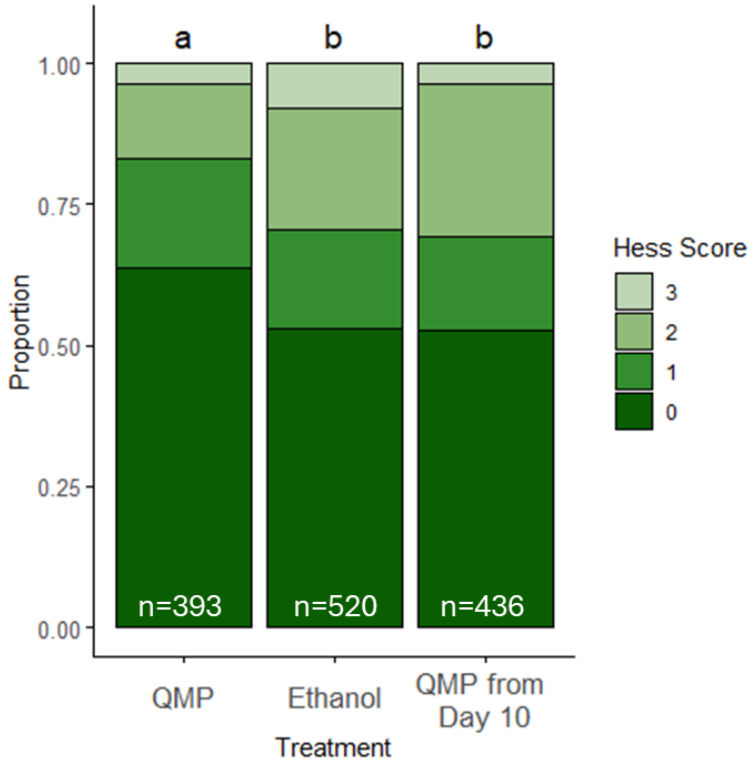


Figure 3.8 – Showing a proportional bar chart of the effect on *A. mellifera* worker ovaries of first allowing ovaries to activate before attempting to repress them with the presence of QMP. Ovary activity is measured via modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present). The y-axis shows proportion of ovaries of a given Hess score, the x-axis shows treatment, with 0.1Qe QMP per day, either from the start of the experiment, or after 10 days, and solvent ethanol control. Significance given as letters ($P<0.05$). Once allowed to activate, honeybee worker ovaries cannot be repressed with QMP.

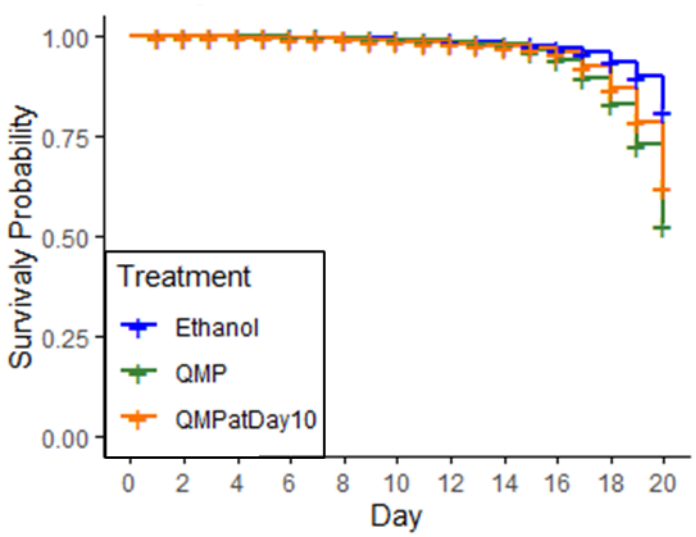


Figure 3.9 – Showing a Kaplan-Meier survival curve of honeybee workers which have been allowed to activate their ovaries before exposure to QMP or solvent control, and a QMP control. The y-axis shows probability of survival, and the x-axis shows time in days. Treatment is given as colour, with QMP exposed bees given 0.1Qe of QMP per day, and an ethanol solvent control, and one group only solvent for 10 days, before QMP exposure for a subsequent 10. There is no significant effect of treatment on survival.

3.3.4.2 *A. mellifera* workers which have become reproductively active *in alvo* cannot be repressed via exposure to QMP

The highly controlled nature of *in cavea* experiments allows us to isolate variables for their independent effect on honeybee biology. However, the synergistic effects of different variables are not tested in these setups. This also tends to produce more false-negative results than false positive, which while useful for characterising fundamental biology, results in a need to test these setups *in alvo*, to confirm the biological relevancy of the variable investigated.

With this in mind therefore, the results of the previous section were repeated with the activation of the ovaries of the bees occurring *in alvo*, in order to investigate whether the activation of the ovaries occurred in a similar manner *in cavea* as *in alvo*.

A. mellifera workers were therefore marked and placed into a queenless hive for 28 days to activate their ovaries before being taken and exposed to QMP *in cavea* for 10 days. (detailed in Figure 3.10)

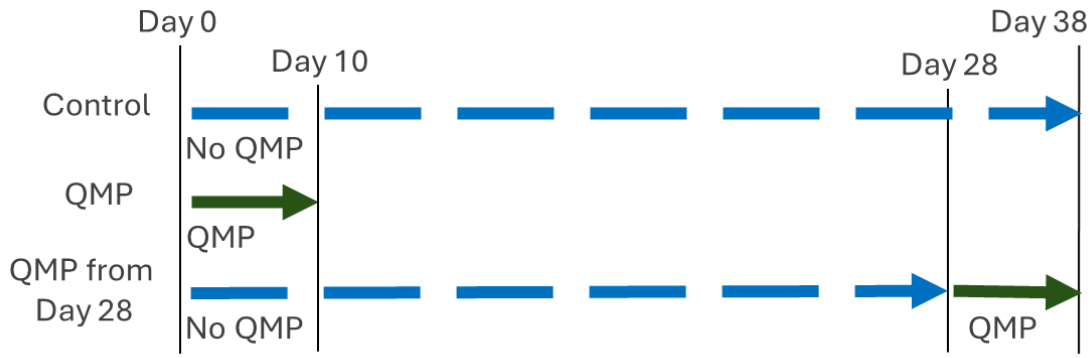


Figure 3.10 – Showing the scheme of the experiment investigating the ability of QMP to switch off active *A. mellifera* ovaries, activated *in alvo*. Cages with bees exposed not exposed to QMP are shown with dashed blue lines. Cages with bees exposed to 0.1Qe QMP per day are shown with solid green lines. The bees in the "QMP from day 28" cages were initially allowed to activate *in alvo* in a queenless hive before being moved to cages for QMP exposure. The bees in the "Control" cages were kept for 28 days *in alvo* alongside the bees in the treatment group, and then moved *in cavea* at day 28. The QMP cages are as in other experiments: exposed to QMP for 10 days before dissection, so as to provide context of a repressed phenotype.

These data are shown in Figure 3.11 (χ^2=23.578, df=2, P<0.001), where the workers which have been exposed to QMP after having activated their ovaries exhibit the same degree of ovary activity as the ethanol control (results of individual pairwise test results in Table 24, in appendices).

There is also no effect of mortality between treatments in this experiment, as can be seen in Figure 3.12, with individual pairwise comparisons in Table 25, in appendices.

The lack of *A. mellifera* worker plasticity *in cavea*, therefore, is not as a result of the *in cavea* environment during the activation period.

There is a possibility that the sampling bias mentioned in section 3.2.1.3 affected these results by virtue of selecting for those bees which are unwilling to horizontally migrate between hives (and so perhaps selecting for those bees which are most likely to lay). The only way to avoid this without introduction of workers exposed to queen pheromones would be to isolate the hives, which we were not able to do due to practical limitations.

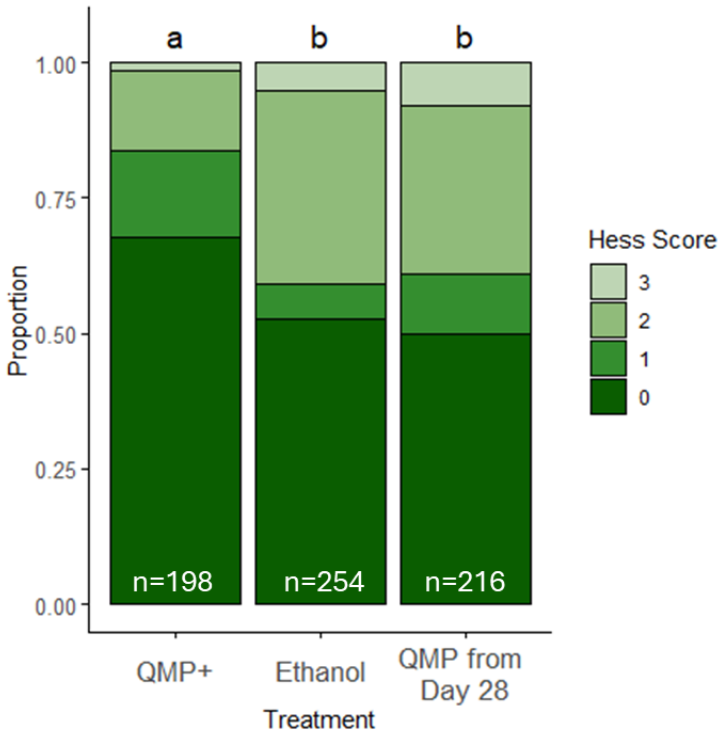


Figure 3.11 – Showing a proportional stacked bar chart of the effect of QMP exposure on ovary activity in *A. mellifera* workers, which have been allowed to activate their ovaries *in alvo*. Ovary activity was measured using a modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present). The y-axis shows proportion of ovaries at a given Hess score, while x-axis shows treatment, with 0.1Qe of QMP per day, or an ethanol solvent control, the last bar is of bees allowed to activate their ovaries for 28 days *in alvo* before exposure to QMP *in cavea* for 10 days. Significance given as letters ($P<0.05$), calculated via CLMM and post-hoc Tukey pairwise test. Honeybee workers are not able to activate their ovaries once they have been activated via exposure to QMP.

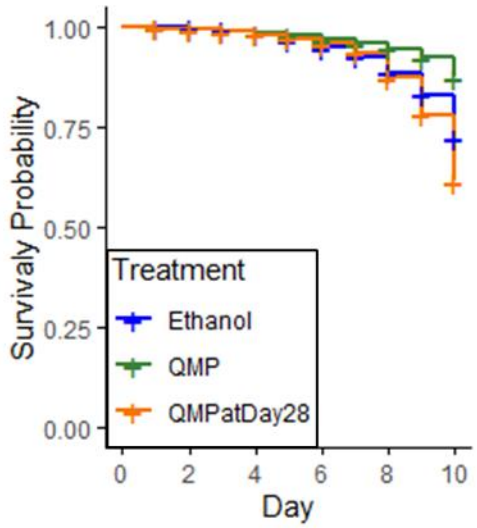


Figure 3.12 – Showing a Kaplan-Meier survival curve of honeybee workers which have been allowed to activate their ovaries before exposure to QMP or solvent control, and a QMP control. The y-axis shows survival probability and the x-axis day of experiment, treatment group is given as colour. There is no statistically significant difference between survival of bees exposed to different treatments.

3.3.4.3 Different stably expressed reference genes produce different trends for gene expression in *A. mellifera* brains of the socially indicative *for* and *dop2* genes

It is possible that the lack of plasticity seen in adult *A. mellifera* workers is as a result of the lack of comb and brood presence in the colony. It is known that in the absence of brood, honeybee workers will switch to foraging roles in larger numbers (246). It is possible that *in cavea* due to the absence of brood pheromones and nursing tasks, the bees change their roles towards foragers.

This social aging, in contrast with their biological aging, manifesting as precocious foraging, might be reflected in their physiology such that they lose the ability to switch on their ovaries in the absence of QMP. This lack of sensitivity to QMP is naturally necessary otherwise honeybee workers not exposed to QMP as they forage *ex alvo* would otherwise switch on their ovaries. This is potentially as a result of the loss of developmental plasticity that generally occurs with social aging in animals (247). It could also be a specific physiological change which occurs in response to aging in the honeybee, as it is also known that workers do not engage in the queen retinue response (which is mediated by QMP (108)) past day 6 in age (248, 249), suggesting that there are changes to the ability of workers to sense QMP as they age.

Published research has shown that expression of both the *for* gene (250) and the *dop2* gene (251) in the brain of honeybee workers positively correlate with biological age, as measured via social role (i.e. that age correlates with social role, and social role correlates with *dop2* signalling). As such, by taking bees of different roles *in alvo* and comparing the expression of these genes to those reared *in cavea* we should be able to identify whether the workers in our cages are precociously becoming foragers, and so losing plasticity of development of their ovaries in response to QMP. Samples were separated by inactive/active ovaries and treatment.

First, in order to investigate the gene expression of our samples, we performed a reference gene analysis using our pooled brain samples. This is necessary as the importance of reference genes, often overlooked in studies utilising RT-qPCR, determines the strength and reliability of our analysis (252). The genes picked as candidate reference genes are a mixture of those used for showing the importance of these two target genes, and also other papers looking into stable reference genes for *A. mellifera* brains.

As can be seen in Figure 3.13, the stability of all of candidate reference genes was relatively high. The threshold for homogenous tissue has been shown to be 0.5 M score (253), and all of the proposed reference genes were below this value (notably so was the target *for* gene).

As a result of this the two most stable genes *rpl32* and *rps18* were deemed appropriate as references for final analysis.

As can be seen in Figure 3.14**B** and Figure 3.15**B**, there is no statistically significant difference in expression of either *dop2* or *for* across samples(13**B**:F=1.0348, df=34, P=0.42; 14**B**:F=0.6963, df=34, P=0.6542; results of pairwise tests in Table 28 and Table 29). This indicates that, both *in alvo* and *in cavea*, our bees do not possess the differences in expression reported for these genes with age in the literature.

The other panels of these graphs demonstrate the importance of choosing appropriate reference genes, and of using more than one during analysis, and represent samples of the variation of results that different “acceptable” reference genes can cause. As can be seen in Figure 3.14**A**, when using *ef1-α* as a sole reference gene the trend (though not statistically significant with the n=6 for each boxplot) of *dop2* expression seems to be that as the bee ages expression of this receptor decreases in the brain (results of individual pairwise tests in Table 27, in appendices), but this same trend is the opposite when using *rpl32* as a reference, as seen in panel **C**.

The same issue exists within Figure 3.15 as well, where in panel **A** we see a potential negative correlation with age and *her* expression when using *ef1-α*, but the opposite trend in panel **C** using *rpl32* as a reference gene.

These four panels were selected out of all the possible reference combinations and individual genes, as they highlight the heterogeneity of the dataset with different reference genes, and the issues that this causes with interpretation of the data as a whole. Particularly as these reference genes have all been used in the literature for *A. mellifera* brain RT-qPCR experiments, with most also being tested for in reference gene analysis papers.

The paper which demonstrated the correlation between aging and expression of the *for* gene did not produce a reference analysis, and used the 18S RNA of the ABI kit as a reference gene (254). The paper investigating the role of *dop2* did not do so using RT-qPCR and so did not use reference genes (251).

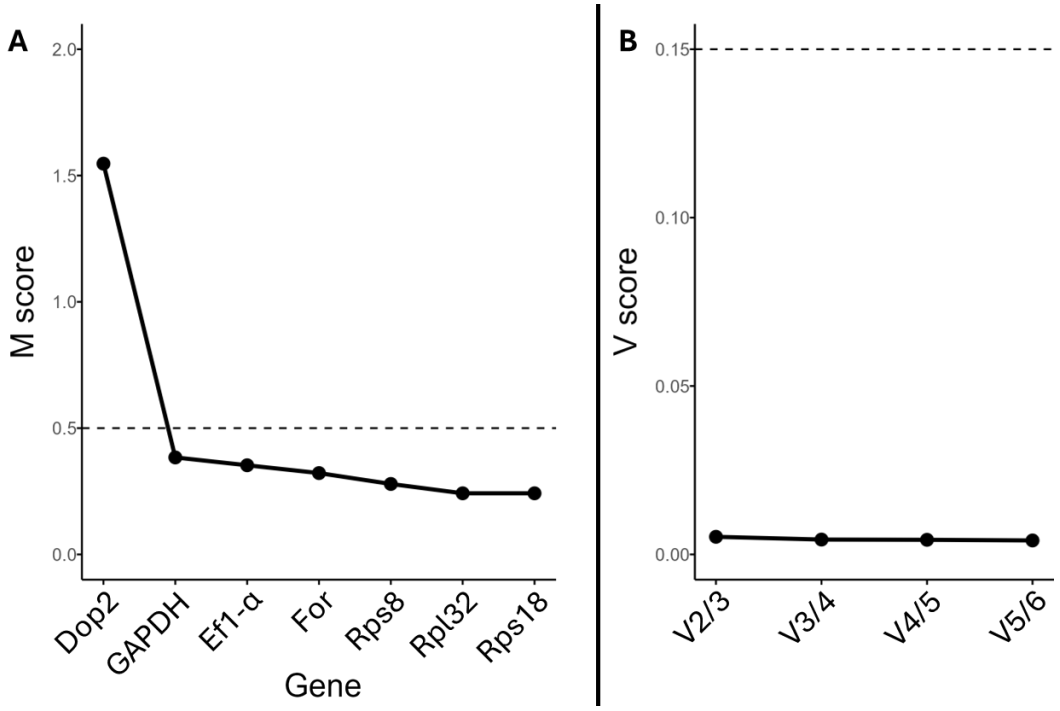


Figure 3.13 – Showing a linegraph of the stability of different potential reference genes for investigating brain gene expression for pools of five *A. mellifera* worker brains. **A**: The y-axis shows M score, a measure of the stability of a given reference gene calculated via Genorm2, while the x-axis shows the gene of interest. A dashed line shows the M-score of 0.5, given as the maximum for reasonable stability of a homogenous cell population (253). All genes but dop2 showed acceptable stability. **B**: the y-axis shows V score, a measure of the stability of combined groups of reference genes calculated via Genorm2, while the x-axis shows increasing groups of reference genes. A dashed line shows the V score of 0.15, the maximum for reasonable stability for a given set of references. All groupings showed acceptable stability.

Unfortunately, as a result of the lack of consistency of trends between mathematically appropriate references, RT-qPCR in this experiment was unable to bring about a reasonable proof for the hypothesis that precocious foragers reduce their plasticity to QMP-mediated repression of ovary activity in *A. mellifera* workers. However, it does raise interesting modes of critique for the use of this technique, and the manner of analysis of reference genes for this species and tissue.

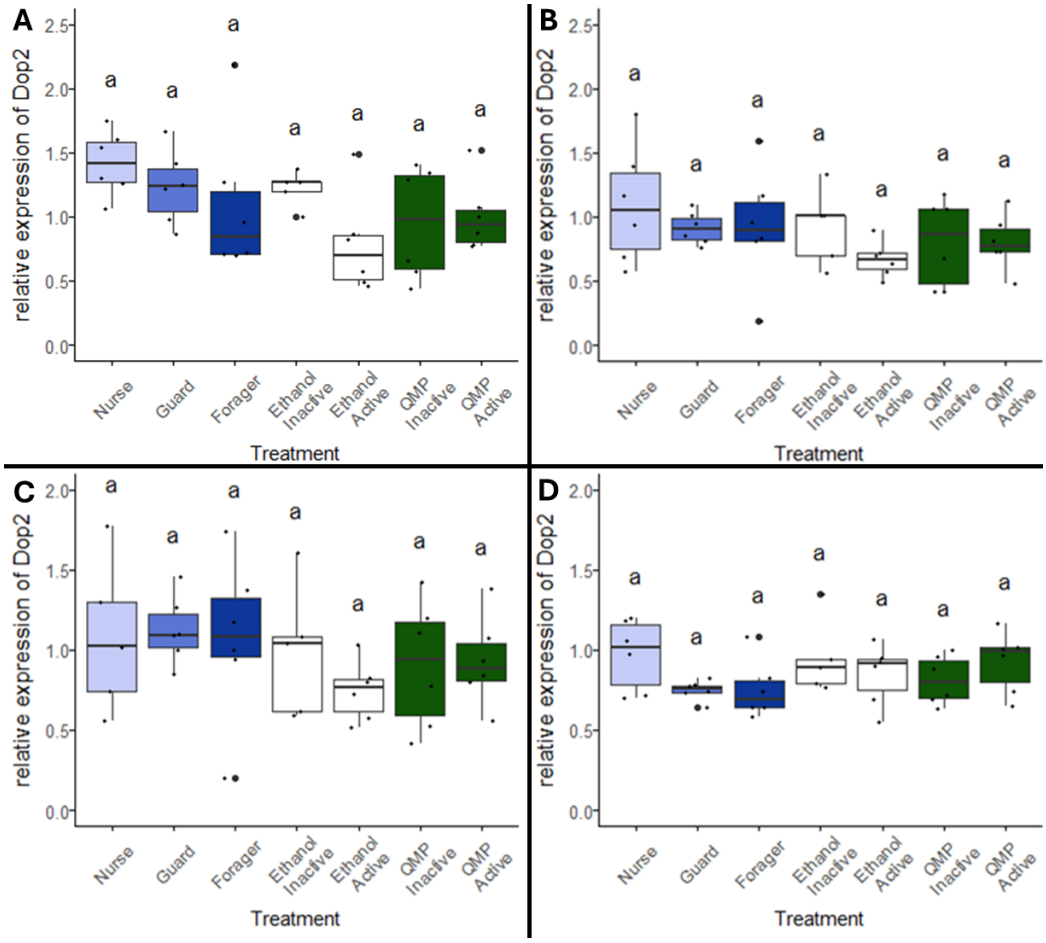


Figure 3.14 – Showing boxplots of relative dop2 expression of worker *A. mellifera* brains across the target samples using difference reference genes. **A**: Ef1α; **B**: Rpl32 and Rps18; **C**: Rpl32; **D**: GAPDH. The y-axes show relative expression of dop2, while the x-axes show the different origins of tissue for treatment groups of the experiment: tissue collected from wild-type nurse, guard, and forager brains, or *in cavea* bees exposed to either 0.1Qe QMP per day, or ethanol solvent control. All samples were pooled groups of five brains, with the inactive and active being five brains from bees with ovary Hess scores of 0-1 or 2-3 respectively. Significance given as letters ($P<0.05$) calculated via GLMM and post-hoc Tukey pairwise tests. The difference in expression seen across these panels shows the importance of good reference gene selection.

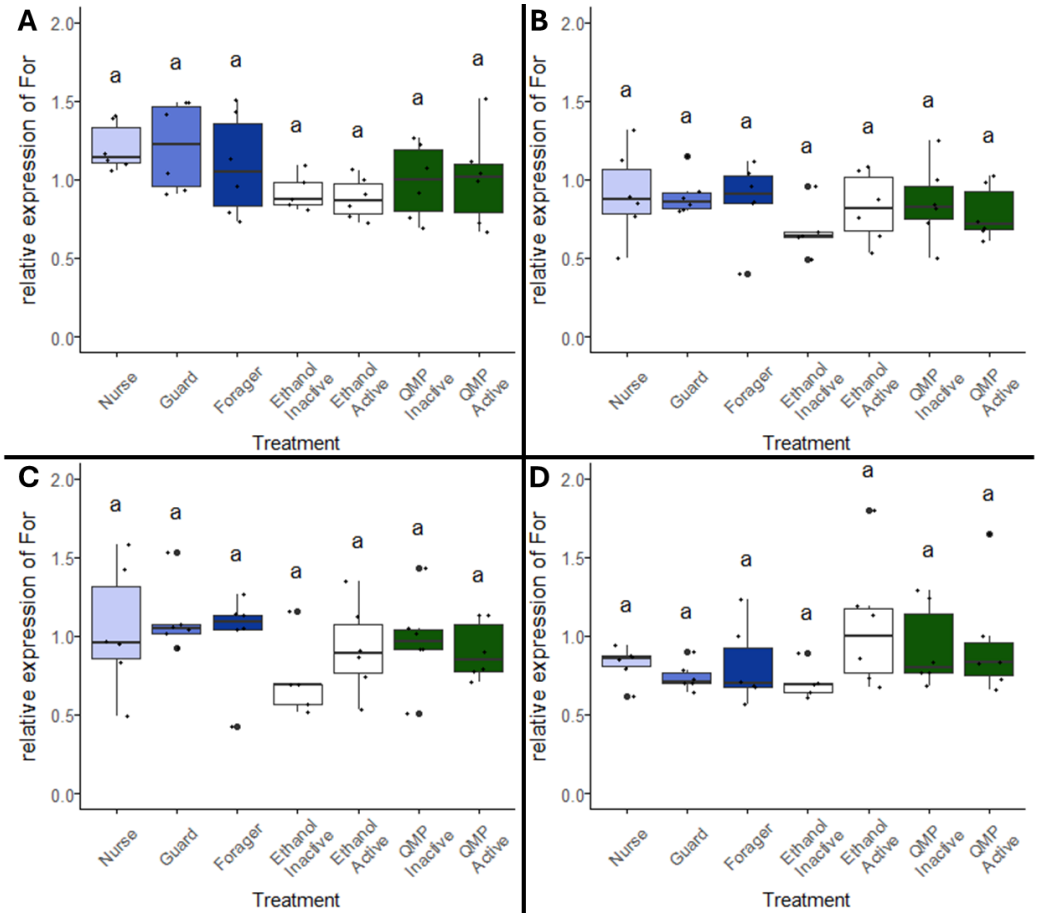


Figure 3.15 – Showing boxplots of relative for expression of worker *A. mellifera* brains across the target samples using difference reference genes. **A**: Ef1α; **B**: Rpl32 and Rps18; **C**: Rpl32; **D**: GAPDH. The y-axes show relative expression of her, while the x-axes show the different origins of tissue for treatment groups of the experiment: tissue collected from wild-type nurse, guard, and forager brains, or *in cavea* bees exposed to either 0.1Qe QMP per day, or ethanol solvent control. All samples were pooled groups of five brains, with the inactive and active being five brains from bees with ovary Hess scores of 0-1 or 2-3 respectively. Significance given as letters ($P<0.05$) calculated via GLMM and post-hoc Tukey pairwise tests. The difference in expression seen across these panels shows the importance of good reference gene selection.

3.3.4.4 QMP signalling period

Given the fact that QMP-mediated repression of worker ovary activity is only able to act as a repression of ovary development, rather than a suppression of active ovaries, then there must be a period of time in which QMP is able to act.

In this mode, the presence of QMP before the onset of this period, and the presence of QMP after the end of this period would fail to bring about repression of ovary activation in *A. mellifera* workers.

3.3.4.4.1 A. mellifera workers demonstrate an intermediate ovary activation phenotype when exposure to QMP is delayed

To identify the beginning of this period, we can begin to expose the workers to QMP at later times. If ovary repression is still able to occur despite the later introduction of QMP, then the QMP exposure was begun at a point before the onset of this crucial period. (detailed in Figure 3.16)

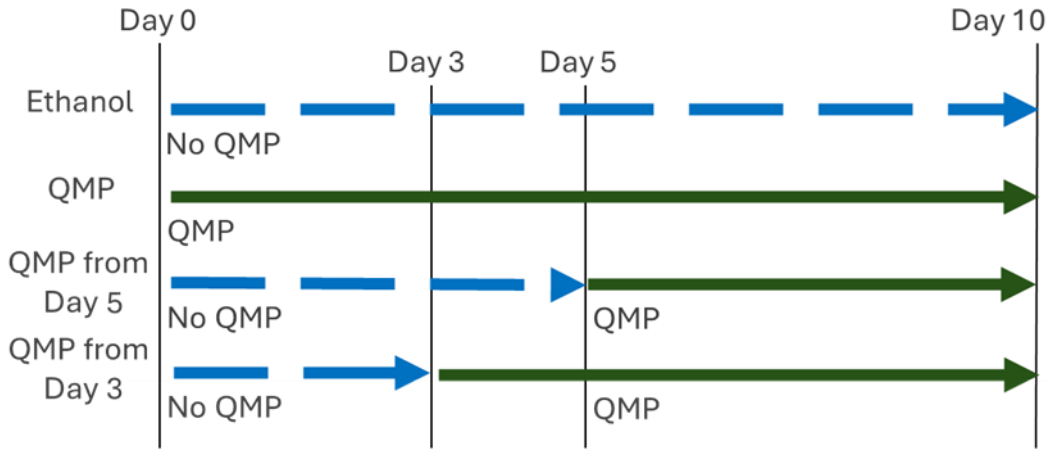


Figure 3.16 – Showing the scheme of the experiment investigating the beginning of the QMP-activity period in *A. mellifera* workers. Cages with bees exposed to ethanol are shown with dashed blue lines. Cages with bees exposed to 0.1Qe QMP per day are shown with solid green lines. For each repeat a cage was exposed to ethanol only, QMP only, or they were initially exposed to only ethanol solvent for three or five days, and then exposed to QMP for the remainder of the 10 days.

As we can see from Figure 3.17 there is a significant effect of treatment on ovary activity (χ^2 = 18.683, df=3, P<0.001). The workers which had begun to be exposed to QMP during the experiment showed somewhat of an intermediate phenotype between the two controls (though statistically the bees exposed to QMP from day 5 were the same as those exposed to QMP the entire time, this is potentially reflective of the over-emphasised role of modified Hess score 0 ovaries in the analysis compared to Hess score 1 ovaries; when observing the score 3 and 2 ovaries we see a far clearer trend. I.e. when observing only active/inactive ovaries as measured by yolk deposition, or by observing the distinction between presence/absence of eggs, the trend is far clearer).

An intermediate phenotype indicates that there is not a hard-wired period in which the bees as a whole are susceptible to the action of QMP, but rather that each individual bee has a period in which it is most susceptible, and that this varies between individual bees. In this scheme we would expect to see a continuous intermediate phenotype. That the latest a worker can be

given QMP and still result in ovary repression *in cavea* is after day five is surprising, as it had been observed prior that the first active ovaries (of modified Hess score 2) being to appear in a cage at day six (observed during section 3.3.5).

There is no effect of these treatment groups on the mortality of the bees, as shown in Figure 3.18, with pairwise comparisons in Table 31 in appendices.

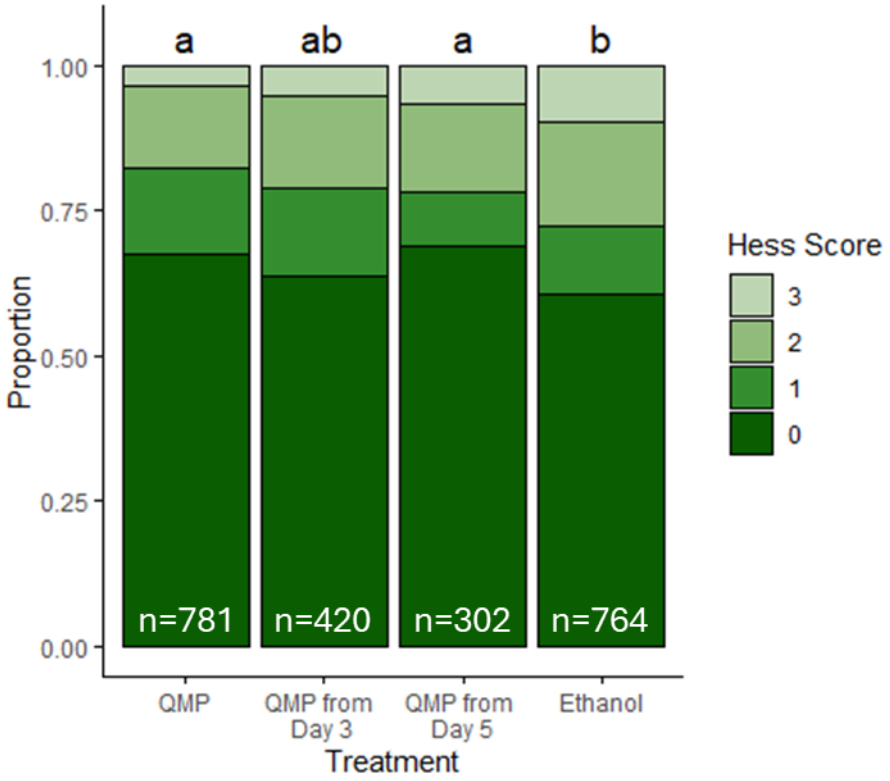


Figure 3.17 – Showing a proportional stacked bar chart of ovary activity of *A. mellifera* workers which have been allowed to activate their ovaries before exposure to QMP. Ovary activity was measured via modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present). The y-axis shows the proportion of ovaries with a given Hess score, while the x-axis shows treatment: either QMP at 0.1Qe per day, ethanol solvent control, or ethanol control followed by 0.1Qe QMP starting at day three or day five. Statistical significance given as letters ($P<0.05$) calculated via CLMM and post-hoc Tukey pairwise test. The prevention of ovary activation via QMP shows intermediate phenotypes between the two controls, when QMP administration is delayed.

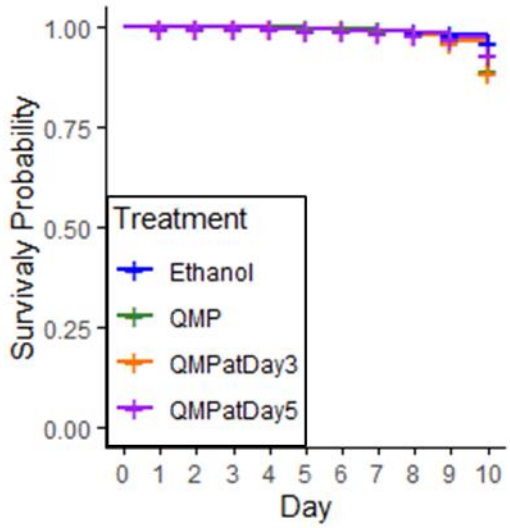


Figure 3.18 – Showing a Kaplan-Meier survival graph of *A. mellifera* workers exposed to QMP at delayed intervals. The y-axis shows survival probability, and the x-axis day of experiment, with different treatment groups given as colours. There is no difference in survival between treatment groups.

3.3.4.5 *In cavea A. mellifera* experiments do not provide enough survival probability with age to provide conclusive evidence of end of QMP-sensitivity period

It has been shown that workers become unresponsive to QMP at later ages (via queen retinue response) (226). Additionally, that foragers must spend large amounts of time away from a hive does not result in their ovaries switching on suggests that once a certain biological or social age has been reached, honeybee worker ovaries are no longer able to be switched on.

In order to investigate this idea, we can expose *A. mellifera* workers to QMP for a given amount of time before removing the QMP to see if the ovaries are able to switch on after QMP has been removed. (detailed in Figure 3.19)

In our *in cavea* setup on an FandP diet, the upper limit of time in which adult workers are able to be kept before prohibitive mortality is 20 days, and with a given 10 days for maximal ovary activation, we are limited therefore to exposing the bees to QMP for 10 days before removal at the latest point.

During the course of this experiment there was particularly high mortality of bees in the QMP cages, resulting in lower than desired samples sizes. This could also have disrupted the results.

Despite this, the data shown in Figure 3.21 shows a significant difference in ovary activity between the two controls, and treatment was shown to be a significant predictor of ovary activity (χ^2=23.929, df=4, P<0.001).

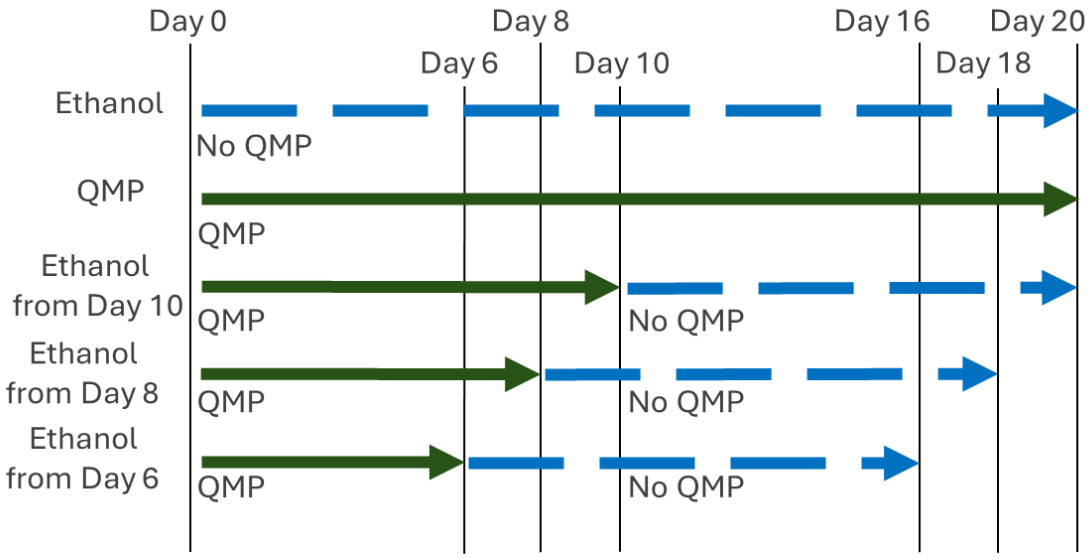


Figure 3.19 – Showing the scheme of the experiment investigating the end of the QMP-activity period in *A. mellifera* workers. Cages with bees exposed to ethanol are shown with dashed blue lines. Cages with bees exposed to 0.1Qe QMP per day are shown with solid green lines. For each repeat, a cage was exposed to ethanol only, QMP only, or they were initially exposed to QMP for six, eight, or ten days, followed by exposure to only ethanol for a subsequent ten days.

All of the cages of bees exposed to QMP and then allowed to activate demonstrated an activated phenotype, which can be seen as none are statistically different from the ethanol solvent control. The data showing the bees which had been exposed to QMP for six and eight days respectively, also showed significant difference to the QMP positive control. The day 10 samples did not differ in a statistically significant way to either control. This could be due to the lower sample size of this treatment type (due to increasing mortality with age, shown in Figure 3.20) resulting in the sample sizes being too low. The results of individual pairwise tests for Figure 3.21 are in Table 32, and for Figure 3.20 in Table 33, in the appendices.

Two different conclusions can be drawn by the lack of statistical significance to either control of those bees exposed to QMP at day 10. Either that this is an indicator of too low a sample size for correct resolution, or that we are observing an intermediate phenotype. In the first instance of this result being low sample size, we would say that the lack of efficacy of QMP-mediated repression beyond the presence of QMP implies either that QMP alone is not able to produce long-term repression of *A. mellifera* worker ovaries, or that the amount of time needed for QMP exposure is beyond the limits of what is possible in this *in cavea* method of investigation.

If the latter is true, and that this sample in which the bees are exposed to QMP for 10 days and then allowed to activate genuinely represents an intermediate phenotype, then the end of the

QMP sensitivity period would begin to occur around the 9-10 day range. We would also expect to see a great deal of individual variation in this period, as we do for the beginning of this period, and so we should also expect to see intermediate phenotypes.

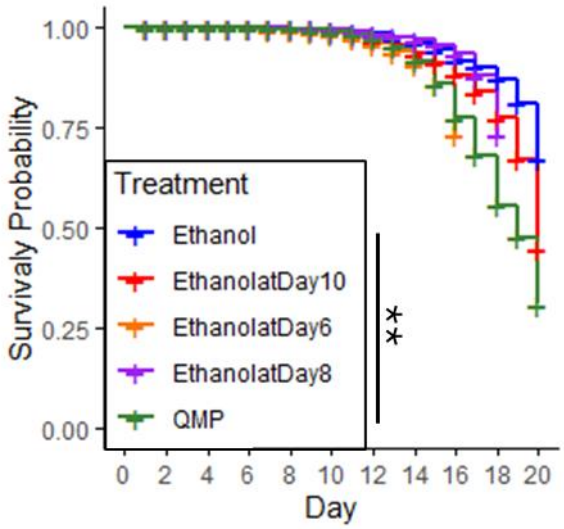


Figure 3.20 – Showing a Kaplan-Meier survival curve of *A. mellifera* workers exposed to QMP and then treatment taken away. The y-axis shows survival probability, while the x-axis shows length of experiment. Colour denotes treatment group. Significance given as **=P<0.01 calculated via cox mixed effect model and post hoc Tukey pairwise test. There was significant difference in survival between positive and negative controls, but no other group.

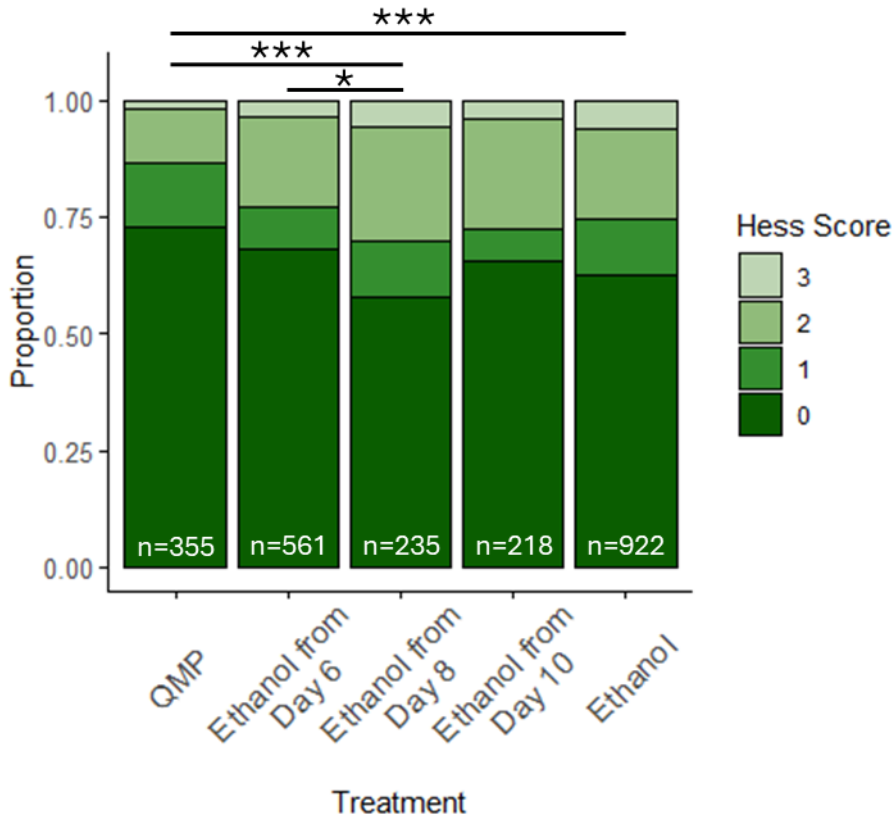


Figure 3.21 – Showing a proportional stacked bar chart of ovary activity in *A. mellifera* workers which have been repressed in their ovary activation before removal of QMP after given amounts of time. Ovary activation is measured via modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present) of ovaries extracted 10 days after removal of QMP, or at day 20 otherwise. The y-axis shows the proportion of ovaries of a given Hess score, while the x-axis shows treatment group: 0.1Qe of QMP per day as a positive control; and ethanol solvent control; or three different treatment groups which have been exposed to QMP at 0.1Qe per day for the given number of days (six, eight, or 10), before being switched to ethanol solvent control. Significance shown as *=P<0.05, ***=P<0.001, calculated via CLMM and post-hoc Tukey pairwise test. Broadly bees exposed to QMP and then allowed to activate showed intermediate phenotypes between the two controls.

3.3.5 Order of Hormone signalling in response to QMP

It has been proposed that QMP-mediated reproductive repression of *A. mellifera* workers occurs via the sensing of QMP at a gustatory/odorant level, before changes in brain chemistry and subsequent changes in internal signalling within the bee (103). In this model, endocrine pathways such as insulin, ecdysone, and juvenile hormone, and neuroendocrine pathways such as dopamine and octopamine, are all possible pathways through which a signal between the brain and the ovary could take place. Notch signalling has also been shown to be involved in the ovary as a signalling mechanism in QMP-mediated reproductive repression of honeybees.

By investigating the gene expression of ovaries, fatbody, and brains of honeybee workers over time, we can identify the sequence of these signalling to elucidate the nature of the possible signalling pathways of QMP-mediated repression of ovaries in this species.

In order to investigate these pathways and their responses to QMP, we must first overcome a significant problem of the biology of the bees which has been evident throughout this chapter so far, which is that in any given cage of bees, even bees which have been allowed to activate their reproduction *in alvo*, at least 50% of all bees will not under any circumstances activate their ovaries. Additionally, *in cavea* regardless of treatment, between 15-25% of bees will always activate their ovaries. This is likely an effect of the colony's ability to maximise its fitness in queenless contexts.

When investigating the impact of signalling pathways in response to QMP therefore, we only wish to investigate those ovaries which have the opportunity to activate if not exposed to QMP, but which do not in the presence of QMP, which is maximally, only roughly 35% of a given set of bees, and in some cases as low as 15-20%. This of course cannot be done retroactively, as in order to categorise the ovary activation of a given bee, we have to extract the ovary, killing the bee in the process.

In order to circumvent this problem, we proposed the use of RT-qPCR using individual honeybee ovaries. In this methodology, any differences between these three groups of bees should be evident in the clustering of data. This would also then provide us with potential genetic markers which can overcome the limitations of observations of ovary score as a macro-anatomical marker.

As has been discussed in section 3.3.4.3, choice of reference gene is important for contextualising any RT-qPCR data, as a result a series of genes were selected as potential reference genes and a reference gene analysis was undertaken, the results of which can be seen in Figure 3.22.

Previous work showing the role of Notch signalling in ovary activity in *A. mellifera* workers found that *mrpl44* and *rpn2* displayed the strongest stability (132). However, in these individual ovary samples, they displayed some of the lowest stability, possibly indicating that there is a significant effect of the three different honeybee worker types (those which activate, those which never do, and those which only do if not in the presence of QMP), on gene stability. The *rpl1* and *rps8* genes showed as the only two genes with an M-score lower than 0.5 (253), and so these two were chosen as the references.

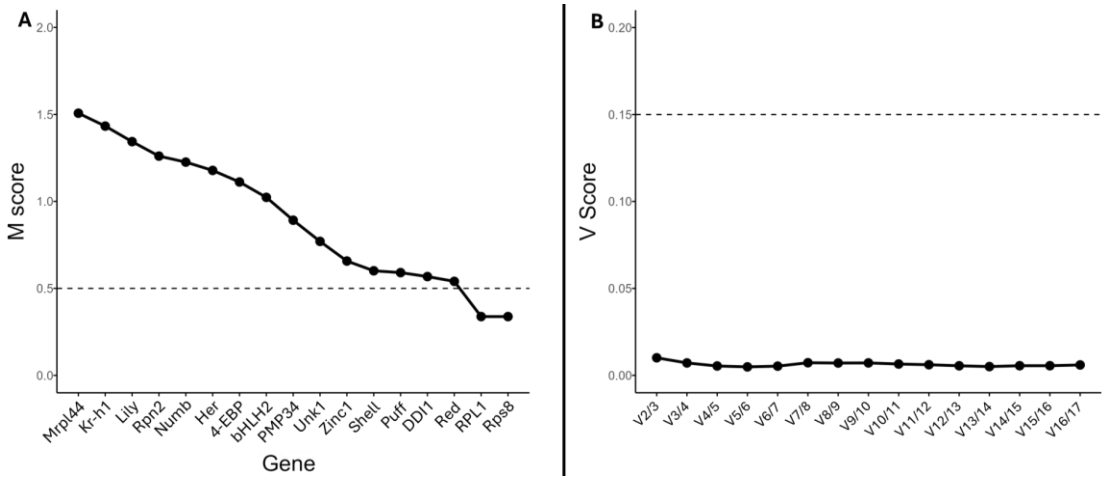


Figure 3.22 – Showing a linegraph of the stability of different potential reference genes for investigating individual ovary gene expression for *A. mellifera* workers. A: The y-axis shows M score, calculated via Genorm2, while the x-axis shows the gene of interest. A dashed line shows the M-score of 0.5, given as the maximum for reasonable stability of a homogenous cell population (253). All genes but Rpl1 and Rps8 showed unacceptable stability. **B**: showing V score of increasing pairs of reference genes. A dashed line shows the V-score of 0.15, given as the threshold below which the number of reference genes used is at an acceptable level. In this case only two reference genes are necessary.

The tissue collected for use in RT-qPCR was of QMP+/- treatment, and samples were collected every two days: days zero, two, four, six, eight, and ten.

Tissue collection was carried out twice, once in 2022, and once in 2023, and the QMP treatment of each was verified and is shown in Figure 3.23**A** and **B** respectively. In both instances the QMP successfully resulted in ovary repression (**A**: χ^2=12.124, df=1, P<0.001;**B**: χ^2=22.406, df=1, P<0.001).

Initially, the genes shown by Duncan et al.(132) to be correlated with ovary activity were investigated, shown in Figure 3.24. *bHLH2* and *her* are both associated with the signalling

pathway mediated by the Notch receptor, and *numb* is thought to be a gene associated with the degradation of the Notch receptor. However, the degree of variance present in each treatment group prevents any meaningful observations being made. There appears to be some clustering of data in a way which suggests that signalling of *bHLH2* and *her* fall off in ethanol treated samples but not in QMP samples (which would be consistent with the findings of Duncan et al. (132)), however this is not statistically significant.

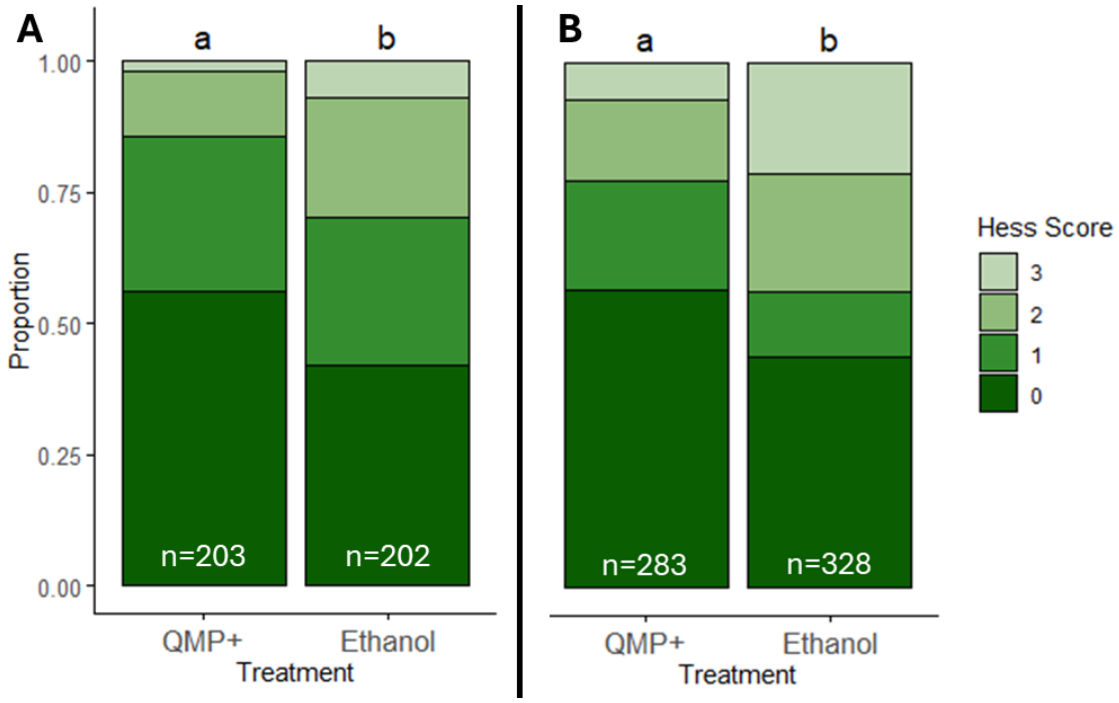


Figure 3.23 – Showing proportional stacked bar charts for ovary activity of *A. mellifera* workers as confirmation of QMP-mediated repression, for two different tissue-collecting experiments. A and **B**, carried out in 2022 and 2023 respectively. Ovary activation was measured by modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present). The y-axis shows the proportion of ovaries at a given Hess score, while the x-axis shows treatment of either 0.1Qe QMP per day, or ethanol solvent control. Significance given as letters ($P<0.05$), calculated via CLMM and post-hoc Chi-squared test. In both panels, QMP-mediated ovary repression was demonstrated.

Signalling pathways such as insulin and juvenile hormone were also investigated, using *4-EBP* which is inhibited as a downstream element of insulin signalling (255); and *kr-h1* which is associated with downstream elements of juvenile hormone signalling (256), but neither of these two genes show any statistically significant difference between QMP and Ethanol treatments at any day.

As mentioned in section 1.3.1.1.5, Ronai et al. have putatively shown that *PMP34* which they have termed *anarchy* is associated with eusocial organisation in *A. mellifera* (257). This gene was therefore also tested but only showed significant difference between the control sample at day 0 and the ethanol sample at day 10, and only weakly so. This indicates that this gene is potentially important in the regulation of ovary activation in worker honeybees, however there was no statistically significant difference between the QMP+/- samples, and so any conclusions drawn from this data are inconclusive.

Of this set of genes, only *her* and *PMP34* indicated any difference between samples, and so these were isolated and shown in Figure 3.26**A** and **B** respectively, however analysis indicates that treatment was not a reasonable indicator of gene expression in *her* ($F=1.939$, $df=44$, $P=0.121$), and although it was for *PMP34* ($F=3.617$, $df=29$, $P=0.016$), this did not result in any degree of statistically significant or relevant observations when pairwise tests are applied. These tests show that there is no statistically significant difference between treatments (results of pairwise tests are in **A**: Table 35 and **B**:Table 36, in appendices).

Given the lack of significance seen in the gene expression of different elements of these signalling pathways, it was decided to attempt to investigate whether any gene was able to produce differential expression in response to QMP versus ethanol, in an attempt to identify if any gene can be used as a predictor for future ovary activation. For these analyses, only samples from day six were tested, as most ovaries in our samples will have activated by day 10, whereas day six showed only marginal activation for a small proportion of ovaries in each cage.

Selection of genes was using unpublished RNAseq data provided by the Duncan lab. This dataset used pools of 50 ovaries of queenless workers with Hess scores 0-3 as defined in this thesis, and compared them to queenright worker ovaries. The workers in this dataset were not age-matched.

This dataset was filtered for expression patterns in which there was a significant difference (greater than 2 standard deviations of the four ovary score's gene expressions combined) in expression of a given locus between ovaries of score 0 (very inactive) and 3 (eggs present), and where only one of these two pools differed from the queenright worker expression patterns. The purpose of this was to find any gene which might act as a marker for active ovary expression in a queenless context, either by high or low expression, but which wasn't simply a result of the lack of a queen (i.e. by filtering out those bees whose ovaries will never activate).

In this way, several candidate genes were selected, shown in Figure 3.25. However, as can be seen in this figure, none of these observations of these genes from the RNAseq dataset were able to be reproduced in individual ovaries.

The conclusion from all of these data, is that the variance of gene expression between individual bees masks any clustering effect that was hoped to have been observed. The normal practice of pooling samples, even as few as five bees per sample, flattens out a great deal of this variance in such a way as to allow broader trends to be seen, and eliminating this pooling seems to increase variance to such a degree that any hoped-for increase in precision is masked.

The high degree of variance seen between individual bees is possibly due to the polyandry that this species exhibits. I.e. that the greater genetic diversity seen as a result of polyandry, produces higher variation in gene networks. (258)

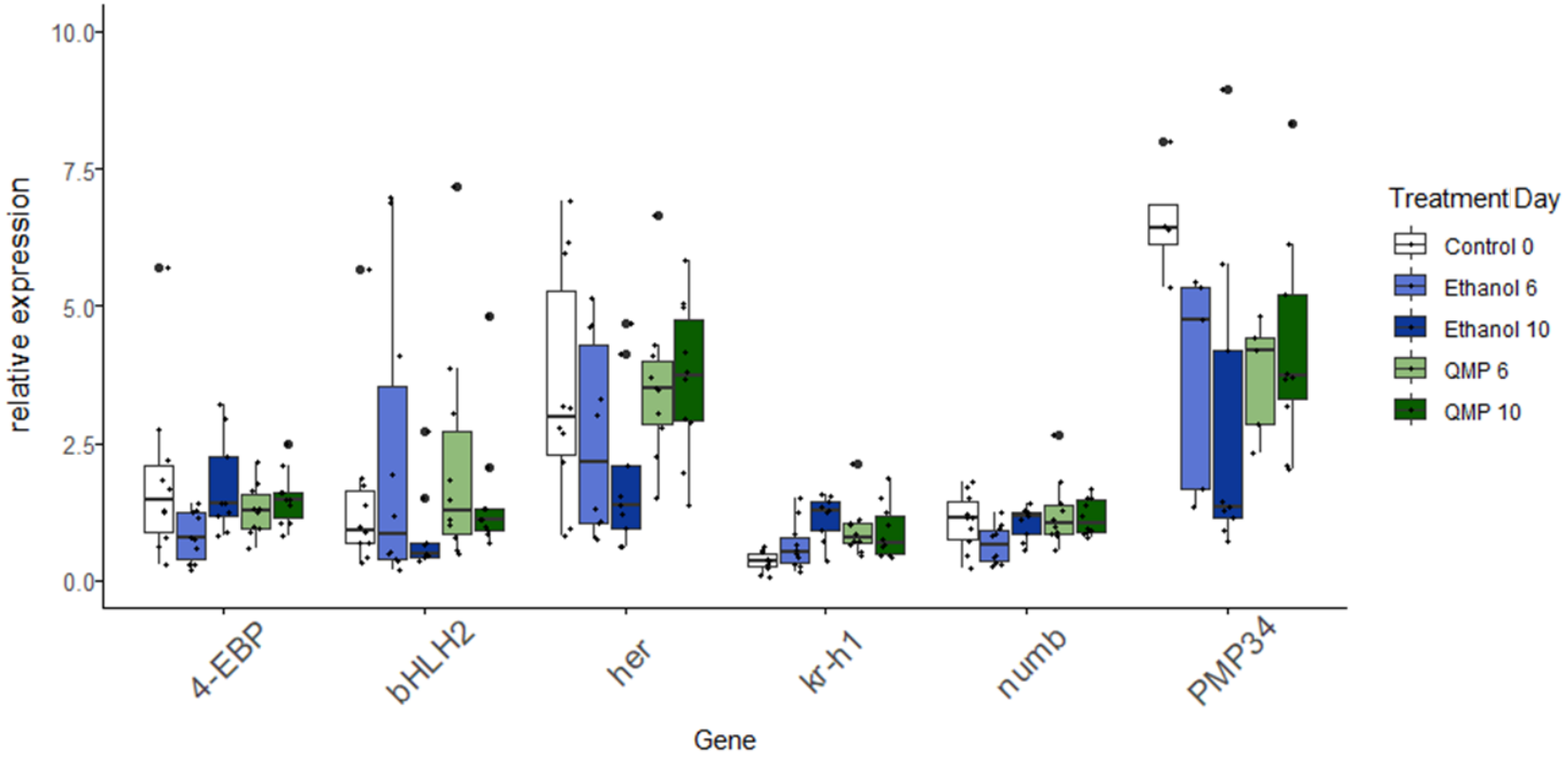


Figure 3.24 – Showing boxplots of relative gene expression of different genes and treatment groups in individual *A. mellifera* worker ovaries. The y-axis shows the relative gene expression of the gene of interest, while the x-axis shows the gene. Each gene investigated also contains five boxplots showing in order of left-to-right: freshly eclosed bees (control 0, white); ethanol solvent control exposed bees at day 6 (ethanol 6; light blue); ethanol solvent control exposed bees at day 10 (ethanol; blue); 0.1Qe QMP exposed bees at day 6 (QMP 6; light green); 0.1Qe QMP exposed bees at day 10 (QMP 10; green). In each case, it was looked-for that the control 0 sample was the same as one but not both of the treatments at either day. This occurred in this figure for her only.

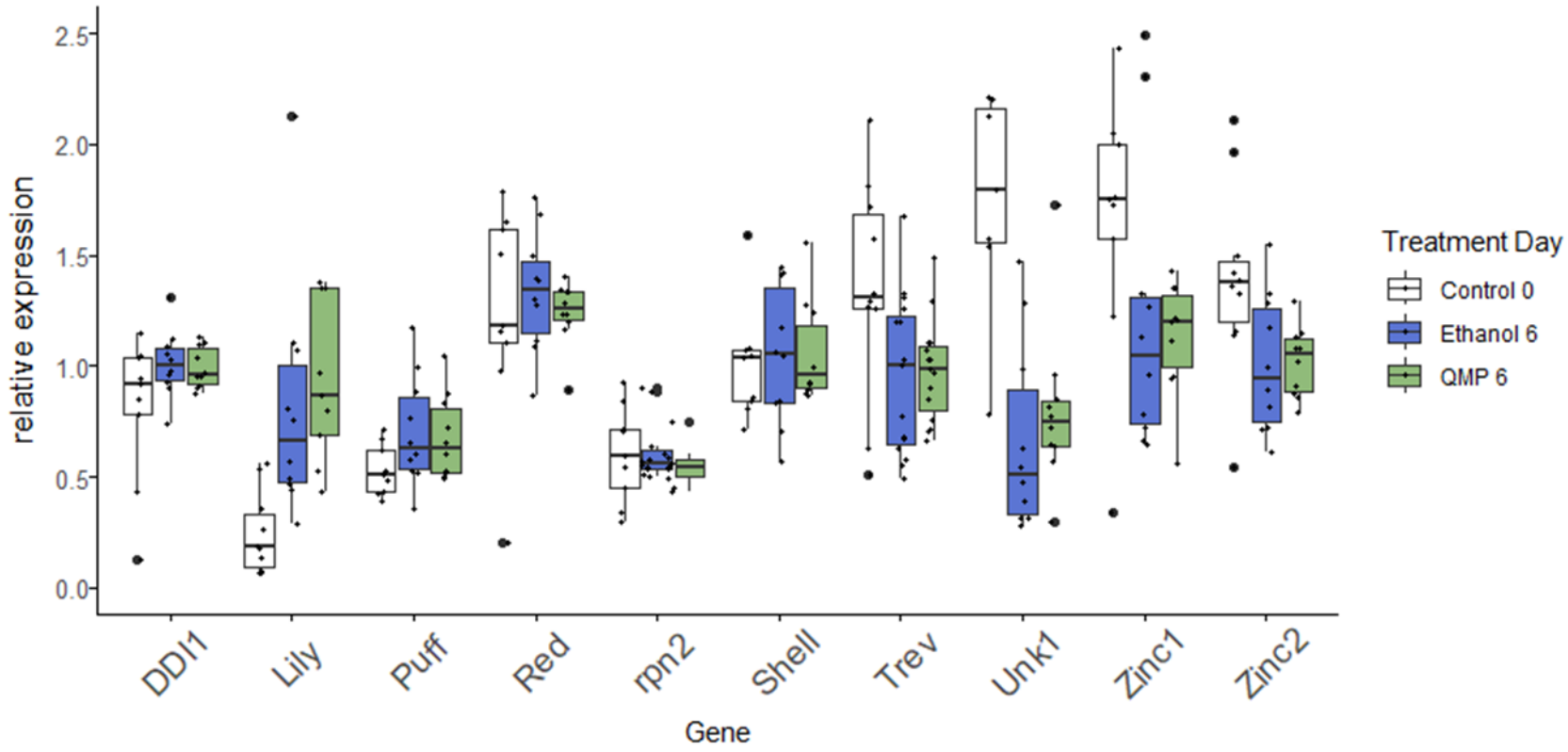


Figure 3.25 – Showing boxplots of relative gene expression of different genes and treatment groups in individual *A. mellifera* worker ovaries. The y-axis shows the relative gene expression of the gene of interest, while the x-axis shows the gene. Each gene contains three boxplots coded by colour of freshly eclosed bees (control 0; white); ethanol solvent-exposed bees at 6 days post-eclosure (Ethanol 6; light blue); or 0.1Qe QMP per day exposed bees at 6 days post-eclosure (QMP 6; light green). In each case, it was looked-for that the control 0 sample was the same as one but not both of the treatments. This did not occur in any of these samples.

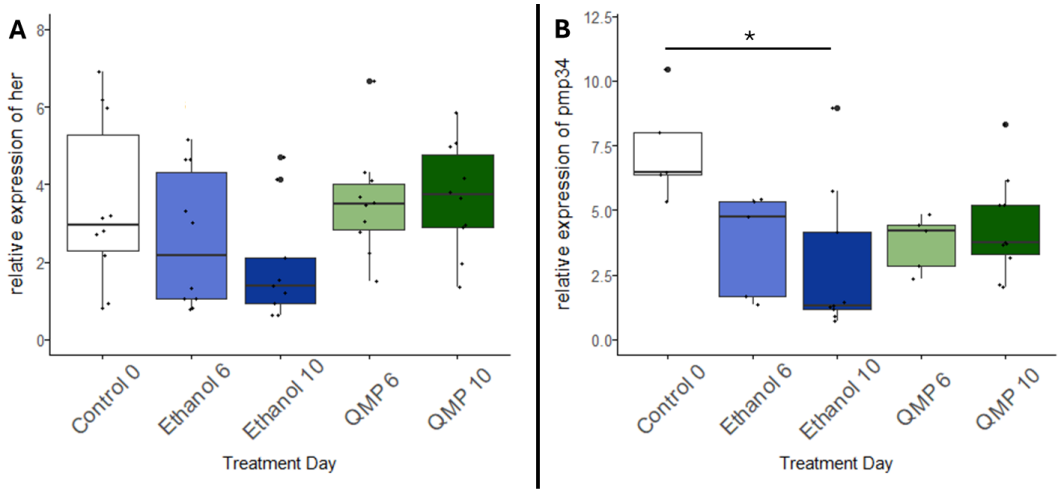


Figure 3.26 – Showing boxplots of the relative expression of A: her, and B: PMP34, over time in individual ovaries of QMP and ethanol exposed bees, taken from Figure 3.24. The y-axis shows relative expression of the given gene, while the x-axis shows tissue collected from: freshly eclosed bees (control 0, white); ethanol solvent control exposed bees at day 6 (ethanol 6; light blue); ethanol solvent control exposed bees at day 10 (ethanol; blue); 0.1Qe QMP exposed bees at day 6 (QMP 6; light green); 0.1Qe QMP exposed bees at day 10 (QMP 10; green). Statistical significance given as *=P<0.05, calculated via GLMM and post-hoc Tukey pairwise test.

3.4 Discussion

QMP's role in reproductive repression of workers and the maintenance of eusociality in honeybee colonies more broadly, has been demonstrated repeatedly. The mechanism of action of QMP is less well understood, either at the molecular level or systemic level within the bee itself.

3.4.1 Diapause as a possible mechanism of QMP-mediated repression of worker ovaries

It has been suggested that *A. mellifera* reproductive constraint may be a form of reproductive diapause (103, 104). This would explain the wide-acting nature of QMP across many different insect species (such as the fruit fly *D. melanogaster*), as reactive repression of ovary activity is an important element of long-term survival. I.e. that in stressful environmental conditions (such as heat stress), or in situations of low food availability, reproduction is a significant drain on stretched physiological resources (make hay while the sun shines, not while it's raining) (197, 259).

In the fruit fly, it has been shown that QMP induces a starvation response (183). The same observation has been demonstrated here in *A. mellifera* whereby QMP-exposed bees consume more carbohydrates than those exposed to just the ethanol control (Figure 3.4), although the protein consumption is not affected by treatment.

This would also be consistent with a specific element of the winter-bee phenotype. During winter-diapause, honeybee workers consume stored honey in order to generate the heat necessary to maintain thermal homeostasis of the hive (52, 188). Their sugar consumption naturally increases due to the increased metabolic burdens which heat production requires (187, 260).

Under any treatment conditions, all honeybee workers consume exclusively pollen at the start of *in cavea* experiments. The lack of ovary activity seen in bees unable to consume pollen shows that it is metabolically essential for ovary activation. The total absence of ovary activation in cages only fed sugar, is notably different to the partial ovary activation *in cavea* in QMP+ conditions. This potentially indicates that starving the bees produces a different phenotype than QMP-exposure (e.g. by inhibited vitellogenin synthesis due to starvation), however this might also be a factor of degree than of nature, whereby prevention of pollen consumption achieves fully what QMP is able to bring about partially. This could be tested by supplying poor quality pollen to the bees, or supplying metabolically inactive protein sources.

It is worth noting that it seems that once this initial pollen has been consumed, the pollen consumption of bees reduces to zero, regardless of if the bees have been exposed to QMP and are therefore repressed, or if they have only been exposed to ethanol solvent control, and so therefore have active ovaries.

It may be that if allowed to lay eggs over longer periods of time than were measured in this thesis, protein needs would increase, and so pollen feeding would increase.

A potential experiment investigating this effect might be possible. By suppling fully drawn-out drone-comb, and allowing the workers to lay eggs *in cavea* over 20 days, in conditions only exposed to fondant after the initial pollen consumption (e.g. fondant only from day seven onwards), we might be able to see the regression of ovaries. This would occur as the bees attempt to lay eggs, but due to the lack of protein cannot supply the base need for vitellogenin synthesis, and so ovaries would regress. This would demonstrate whether ovary activity were still reversible via starvation response.

The gene expression profiles of winter bees are also known to be distinct to the gene expression profiles of worker summer bees (261, 262). A simple experiment therefore comparing the expression profiles of those genes most indicative of the winter bee phenotype, with those of QMP+/- bees *in cavea* would clarify if QMP-mediated ovary repression works through similar mechanisms to that of the winter bee phenotype, and that this form of reproductive repression is taking advantage of these diapause pathways. A similar experiment has been undertaken in fruit flies, showing that cold-induce diapause exhibits specific changes in gene expression of insulin and glucagon signalling genes (199), and these genes are also indicated in the fruit fly response to QMP as a starvation response (183). As a result, these genes are good candidates for investigating the difference in QMP-mediated repression of honeybee worker ovaries, and comparing that to winter-bee ovary expression. Other candidates might also include the Notch-related genes *bHLH2* and *her* which have both been indicated as differentially expressed in QMP-treated honeybees (132).

3.4.1.1 The plasticity of honeybee worker ovary development

A key aspect of diapause in the fruit fly is its reversibility (199). This plasticity allows for rapid changes of activity and reproduction to take advantage of rapidly changing environmental conditions.

The lack of plasticity of honeybee worker ovaries to QMP *in cavea* reported here is therefore surprising. A paper has been published previously showing the opposite conclusion (225), however there were significant differences in methodology between the published paper and

the experiments carried out in this chapter. The number of bees per cage was much lower than in our lab setup, at only 30 bees per cage, comb was present (presumably sourced from a queenright hive), and in order to repress their reproduction, they were placed into a queenright microcolony, which included brood and workers who had been maintained in a queenright setting.

It is known in an apicultural context that once a hive has been dequeened, there is only one opportunity to requeen (roughly two to three weeks), before the colony becomes “hopelessly queenless”. This combined with the evidence of sections 3.3.4.1 and 3.3.4.2 indicate that the biological reality of these organisms is complex, i.e. that there are clearly interacting factors which define latency for plasticity response, and that plasticity is perhaps far more context dependent than assumed at first.

A key difference between the findings of the paper and this chapter may have been the presence of brood, and therefore brood pheromone in the microcolonies used to suppress the reproduction of the workers in the paper. *In alvo* the presence of brood would not occur in the event of a failed requeening (263), and so the presence of brood may induce a degree of plasticity not seen simply by the presence of QMP.

It may also have been as a result of worker policing (whereby workers consume eggs laid by other workers (129)), and although this is discussed in the paper, and for preventing this the workers with activated ovaries were sprayed with sugar water to limit worker-worker aggression, this does not necessarily indicate that no worker policing occurred, just that it didn’t occur within the first hour of introduction. The physical element of reproductive constraint has been observed in honeybees (129), but also in bumblebees (264), and perhaps therefore reflects a more ancestral mechanism of reproductive repression. The fact that the treatment bees were allowed to interact with honeybees that had always been repressed by virtue of the presence of the queen, hints at the fact that these physical social interactions may be as, or perhaps more, important than the role of QMP in mediating reproductive constraint. It has been observed previously that the mechanisms of reproductive constraint in honeybees are highly redundant (47) (presumably as a result of the extreme evolutionary pressure of the maintenance of the eusocial system).

In this way, QMP alone being unable to bring about repression of active ovaries, but the presence of queen, workers, and brood being able to, perhaps suggests several things: that QMP as a queen pheromone is less developmentally fundamental than other holistic forms of social organisation; and possibly that QMP is only able to act fully in the presence of other forms of reproductive constraint.

This thesis primarily investigated the role of plasticity in worker ovaries as a measure of whether the ovaries could be repressed once activated. The observable lack of this plasticity, especially relative to other QMP-sensitive organisms like the fruit fly, hints at a core difference in the mechanism of action of QMP between these two species. Notably, although *D. melanogaster* has demonstrated a plastic response of recovery from QMP-induced repression of reproduction, it has not been tested whether already-active fruit fly ovaries can be repressed with QMP (265).

Whether the difference in plasticity between the QMP-mediated reproductive repression of honeybees and fruit flies is evidence of a sensory exploitation method of evolution, or whether the lack of plasticity seen in honeybees is as a result of changes to the nature of an ancestral signal with the evolution of eusociality is unclear.

The fact that there is a difference of fondant consumption in QMP-exposed bees suggests that the mechanism was perhaps originally the same, and the loss of plasticity for ovary repression in this lineage to the same pheromone indicates either that the mechanism has changed (perhaps that diapause has been coopted for the maintenance of eusociality). It is also possible that the increased fondant consumption of QMP-exposed bees is unrelated to the starvation response triggered by QMP-exposure in the fruit fly.

3.4.1.2 The role of high variation between individuals of a given hive

The large inter-individual variation shown in the results of both the attempts to characterise the beginning and end of the QMP-activity period (in Figure 3.17 and Figure 3.21), and the attempts to characterise the order and nature of the gene expression changes seen during ovary activation, demonstrate that the honeybee hive possesses an ability to adapt to favourable conditions as necessary.

The loss of plasticity that we see in the ability of honeybee workers to switch off their ovaries, and therefore to react to the potential regaining of a new queen, is offset at the colony level by the large variation in individual honeybee responses in other areas. I.e. that the variation in rate of ovary activation and the variation in response to loss of queen, allows for greater plasticity at the colony level, which mitigates any loss of plasticity at the individual level. The inter-individual variation is also surprising with regards to the genetics of honeybees, as they exhibit far lower genetic diversity than other species of comparable numbers (due to the genetic bottlenecking produced by the very small number of reproductive individuals (20)).

This inter-individual variation also makes any experiment attempting to differentiate between the three groups of bees found in cages (those that never activate their ovaries; those that

always activate their ovaries; and those that only activate their ovaries in the absence of QMP) much more difficult. The variation seen in degree of ovary activation *in cavea* is also very high, and is likely a mixture of the natural variation of seasonal effects on worker development and behaviour, and the inter-colony level genetic effects of QMP-mediated repression and ovary activation.

It is known that sensitivity to QMP is a trait which can be manipulated at the colony level, and experiments breeding colonies to low QMP sensitivity have been carried out before (266). It is therefore not surprising that this variation is also seen throughout the experiments undertaken in this thesis, where individual hives contributed greater or fewer amounts of brood to experiments as possible (depending on the strength of the colony and brood availability, which was not consistent throughout the growing seasons).

This inter-individual variation however can also be seen within a given cage of bees: QMP alone is not able to induce reproductive constraint in those bees which always activate their ovaries (10-15% of a given cage), and the threshold for activation is never able to be met for ~50% of the bees. Once we observe that the sensitivity of bees to the repressive signals on ovary development differs between cages, and that individual gene expression varies greatly between workers. The different thresholds of sensitivity to reproductive constraint that the individuals possess results in the variation of ovary activation we see between individuals.

3.4.2 Reproductive plasticity or inhibited development

Whether or not diapause is the mechanism by which ovary repression is able to occur via starvation or QMP-mediation, the assumption has been that this repression is part of an adult plasticity response. I.e. that it forms part of the toolkit of methods that the imago is able to use to respond to changing environmental cues (179, 196, 259).

These environmental cues are of course diverse. They could be related to similar cues as diapause mechanisms, e.g. stress induced by temperature, starvation etc. But they could also be related to important aspects of social organisation.

3.4.2.1 The role of ontogeny in these models

Given that the bees in the experiments reported here lack the plasticity previously reported in the literature, the question of adult plasticity is perhaps brought into question.

The pheromonal and gene expression changes brought about by worker ovary activation, mimic those that occur in the queen (100, 267). From a developmental perspective, although there are marked anatomical differences between the two female castes in *A. mellifera*, the

queen and the workers exhibit similar phenotypes once the worker has activated her ovaries (with the exception of mating and the presence of spermatheca, though in some subspecies of honeybee, such as the cape honeybee *A. mellifera capensis* workers can also possess spermatheca (268)). Any differences in life strategy between the two castes, therefore, have to be understood with ordinary evolutionary principles, one of which is that non-harmful traits do not implement selection pressure on survival.

In this way behaviours and anatomical characteristics of the queen which cause no harm in the workers, but do not necessarily possess any increases to fitness are maintained. At a later point in evolution, these characteristics may be co-opted into an evolutionary strategy.

This may have occurred in the evolution of eusociality in the honeybee.

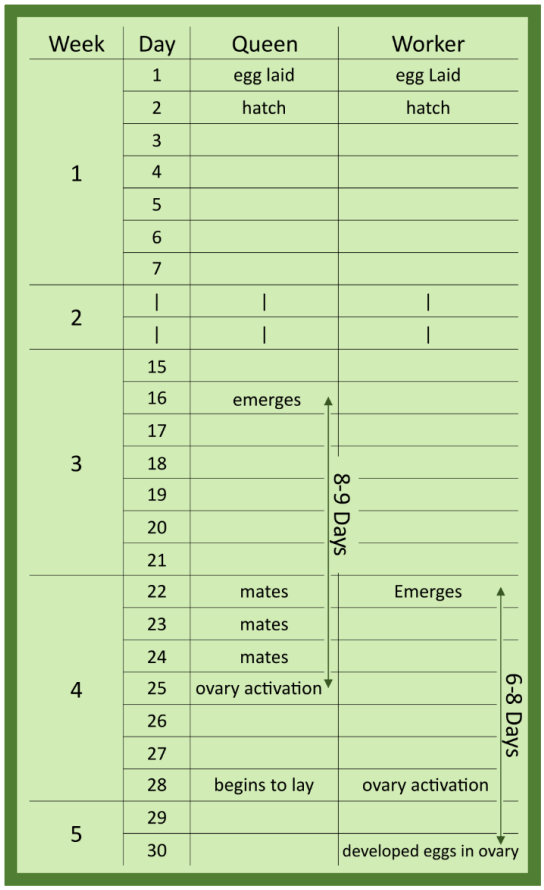


Figure 3.27 – Showing the developmental time of queens and workers. The time between eclosure and ovary activation is roughly the same between workers and queens. This suggests a similar period of zero activity that may have been coopted for QMP-mediated reproductive constraint, via the repression of ovary development similar to a puberty blocker in mammals. Figure adapted from (22).

The life strategy of the honeybee is such that the queen, once she has emerged, must engage in mating flights before returning to the hive to take up residence as the new reproductive (with the existing queen swarming and forming a new hive). As a result of this, she emerges with undeveloped ovaries. This is necessary, as ovaries of honeybee queens are very large and complex, with as many as 200 ovarioles per ovary (22). The metabolic load necessary for activation of these organs is prohibitive to the metabolic load needed for prolonged mating flights. As a result, she does not activate her ovaries until several days after she ecloses, three to four days after she has mated for the first time (22) (with her ovary activation thought to be triggered by that mating, as virgins almost never activate their ovaries (269)).

This period of ovary activity after eclosing is unusual when we compare this species to closely related solitary species, which emerge with active ovaries, in order to maximise the amount of reproduction which can be undertaken as adults (270).

Given that this period exists in queens, it is perhaps therefore unsurprising that this period exists in workers too. It is possible that, as a manner of maintenance of the eusocial system, queen pheromones have evolved to inhibit the development of ovary activation in this period of no ovary activation post-eclosure in the early imago. The role of queen pheromones in honeybee workers would therefore reflect a form of puberty blocker which prevents ovary activation, rather than workers engaging in imaginal plasticity. This would also be a form of reproductive constraint, not an example of honest signalling of fertility. This would explain why the active ovaries of adult workers are unable to be repressed: we are not seeing a plastic imaginal response to ovary activation, but an inhibition of sexual development which, for unrelated reasons, has retarded to early adulthood.

A way of testing to see if the workers do not activate their ovaries as a result of developmental repression, would be to measure the rate of ovary activation under different conditions. Given that QMP exposure does not prevent subsequent ovary activation, one can expose freshly eclosed bees to QMP to prevent ovary activation, then withdraw QMP at a later time (e.g. at day six). The rate at which ovaries begin to develop can be compared to the rate at which ovaries develop in the first ten days post-eclosure in a queenless setting. If the rate is the same then it is likely the ovaries are kept from developing as an inhibition of development. If the rate is more rapid after some days of QMP exposure, then the protein consumption seen at the first few days post-eclosure represents the conclusion in development of the bee, and the QMP can be seen to be repressing the ovary activity in the manner of adult plasticity. If the rate is less rapid after some days of QMP exposure is less rapid, then this may indicate a plastic approach, but one in which plasticity decreases with age.

3.4.3 Feeding effects

During the experiments where QMP was given to bees and then withheld to investigate the potential latter end of a QMP activity period, it was observed that QMP was able to inhibit ovary activation for up to 10 days before ovaries were able to subsequently activate (though not in a statistically significant manner). The ability for worker ovaries to activate does demonstrate a degree of plasticity that could perhaps be investigated in more detail.

Understanding, for example, the implicative difference of feeding effects on ovary activity might provide a better comparison for the nature of pollen-starved ovary repression and QMP-mediated repression.

By performing a similar experiment to that performed before, but instead of using QMP as the repressor of ovary activation, instead using pollen-starvation, then it would perhaps delineate a comparison between the two models of starvation diapause response, or of developmental inhibition. I.e. by only allowing workers to eat fondant for e.g. 10 days, then giving them access to pollen, would they then activate their ovaries in a similar manner to bees not initially starved? If there was a difference in activity produced by this experiment in comparison to bees which had been repressed via QMP then allowed to activate, it would imply a different mechanism between the two.

There are also possible gene expression targets to investigate the possible difference between starvation and QMP-mediated reproductive repression, particularly investigating insulin signalling, and stress-response genes.

3.4.3.1 Alternative Explanation for Difference in Fondant Consumption Between QMP and Solvent Control Exposed Bees

An alternative explanation of the data showing that QMP increases consumption of fondant, is that the other roles of QMP are producing this difference in consumption rather than the role of reproduction repression. This is unlikely however, as these other known effects should decrease consumption, not increase it e.g.: QMP is known to decrease physical activity of workers (115, 240); it is known to inhibit social aging, i.e. prevent forager development, and so prevent resource provisioning for foraging flights (241); it is known to inhibit negative learning, and so therefore reduce brain activity (230). All of these effects would theoretically decrease fondant consumption, and so the effect of increased consumption is not only occurring as a separate mechanism to these presented, but such that it is able to overcome the metabolic surplus produced by the other effects of QMP.

3.4.4 Evolutionary Implications

If we take the lack of reversible plasticity shown in this chapter as an accurate portrayal of the natural conditions in which workers activate their ovaries, then we can perhaps see how elements of the diapause pathways that naturally exist in insects may have been coopted to produce the reproductive constraint necessary for the maintenance of a eusocial system.

A defining characteristic of eusociality is the reproductive division of labour, and in *A. mellifera* this has resulted in the evolution of two very separate developmentally distinct castes. The honeybee is ecologically dominant in its native tropical environment, and dominant in the temperate regions in which its seasonal winter diapause occurs. The evolutionary selection pressures for maintaining this complex system are therefore immense.

The conditions in which worker ovaries would find the need to activate (in hopelessly queenless colonies) are the one life-moment in which it is in the reproductive interest for individual workers to reproduce. Under normal circumstances they obtain greater indirect fitness by caring for their siblings, the brood produced by the far more fertile queen. But once a queen is dead, and attempts to produce another have failed, the hive is doomed also to die. In this moment, the worker can maximise its own fitness (and indeed the fitness of the hive as a whole), by working to produce drones.

It is possible, after the initial failed requeening from within the hive (generally induced by eggs or young larvae being moved into a new queen cup one to two days after the loss of the previous queen), for a queen to be produced. This occurs in apicultural settings fairly easily, as a queen can be supplied from another colony, however it can also occur in nature. When a hive produces new queens for any reason, it produces several. Ordinarily these would return to their hive of origin and fight for dominance. However it is eminently possible, especially when there are multiple hives in a small area, for the newly-mated queen to return to an adjacent hive.

If this hive is queenless, then it is not in the interest of the hive to accept her. The degree of reproductive fitness gained by the workers is minimal, if they are not closely related to the queen.

In this natural setting therefore, the lack of plasticity seen in adult workers, especially after a failed re-queening makes evolutionary sense. In natural conditions there is no possibility to rear a new queen after the first has failed, and so any new queen after this point is very unlikely to be closely related, in which case it is more in the reproductive interest of the hive to continue making drones in order to maximise their fitness, and not to accept a stranger queen.

Notably, even in these conditions, only roughly 50% of the workers will activate their ovaries (Figure 3.11). Presumably this is the ideal ratio for maximising the fitness of a given dying-hive such that there are still workers to care for developing drone brood, but enough workers laying eggs so as to provide as many drones as possible. This could be investigated via theoretical modelling.

3.5 Conclusions

Although the underlying mechanisms of action of *A. mellifera* QMP-mediated repression of ovary activation are still obscure, there have been some useful inroads to further experimentation.

It has now been shown that the plasticity previously assumed honeybee workers were able to possess, is far more complex than has been commonly thought.

The period of activity in which QMP is able to bring about repression in the first week post-eclosure seems to vary a great deal between individual bees, as does the gene expression profiles of individual bees. This inter-individual variation likely allows different colony-wide responses to adverse conditions.

Regardless of treatment, *in cavea* honeybee workers consume exclusively protein-rich foods after eclosing, switching to sugar rich foods after a few days, and then maintaining an exclusively carbohydrate diet after. Additionally, workers exposed to QMP consume greater amounts of carbohydrates than those not exposed, especially just after the switch point between foods.

3.6 Future Work

There are several possible pathways for further research into the nature of QMP-mediated repression.

3.6.1 Understanding the mechanism of action of QMP

Elucidating the mechanism of action of QMP, particularly the role of internal hormone signalling, and neurotransmitter changes in relation to the repression or activation of ovaries is still of paramount importance.

3.6.1.1 Gene expression profiles of workers

Although the experiments using single ovaries showed too great a variance to produce interpretable data, using large numbers of pooled samples would mitigate the effect of the off-target observations which using single ovaries was intended to avoid.

By using much larger numbers of repeats of pooled samples of five bees (in ovaries, fat body, and brain), taken every day in QMP+/- conditions, we can observe the changes in gene expression that occur as the ovaries activate. A large sample size is able to offset the masking effect of the workers which never activate their ovaries, and the workers which always do *in cavea* with QMP exposure, by relying on the assumption that these two groups of bees have similar gene expression profiles between treatment groups, and that this is the reason that they do not produce differential ovary responses to treatment. By comparing these results to the expression profiles seen in the repressed samples, we can identify any changes in signalling of insulin, juvenile hormone, and ecdysone signalling across the bee.

This could be done via RT-qPCR (Directly for insulin signalling elements, though as neither ecdysone nor juvenile hormone can be directly observed using this technique, proxies such as kr-h1 for juvenile hormone, and E75 for ecdysone (271, 272)), however it may also be valuable to initially do so using RNAseq in order to identify the most appropriate target genes. Potential targets are genes of signalling pathways which have been found to show differential responses in QMP-exposed bees, but the specific downstream effects, for example the possible difference between Foxo versus Tor signalling as downstream elements of Insulin signalling have not been characterised at the tissue level in honeybees.

Additionally, in order to investigate the changes in neurotransmitter levels, the brains can be taken and processed using HPLC to identify the relative levels of dopamine and octopamine in the brain, investigating these changes over time and according to reproductive status of the bee.

The results of this should be able to produce a broad map of the signalling pathway changes that occur as the honeybee activates its ovaries compared to QMP-mediated repression.

This experiment can also be repeated using two other mechanisms of reproductive repression: protein-starvation (whereby the repressed bees are prevented from activating their ovaries via the absence of pollen, or by provision of pollen in decreasing colonies (e.g. by using a bulking agent to reduce the protein content of the pollen mixtures), and *in alvo* repression (by marking the bees and returning them to the hive for their reproductive constraint to occur in a more natural setting).

In the former, we would be able to see whether the role of QMP-mediated reproductive constraint occurs at a molecular level, in the same manner as starvation-induced diapause mechanisms.

In the latter, we would be able to see if *in cavea* QMP-mediated repression exhibits the same molecular mechanisms of control as that seen *in alvo*. This is particularly important, as it would allow us to see if there are any additional molecular mechanisms which things such as e.g. brood pheromone, Dufour's gland, worker policing, or other unidentified method of reproductive constraint; can use to produce reproductive constraint, or whether they induce stronger responses of the same mechanisms as QMP. This would help to elucidate the nature of the redundancy of reproductive repression in this species. If the mechanisms are different, or the same, would also indicate how these different redundant systems evolved relative to one another.

3.6.1.2 Winter diapause

The potential inducing of winter-diapause mechanisms could also be tested via the use of gene expression comparisons via RT-qPCR. By identifying gene markers for the onset of winter diapause using RNAseq datasets (e.g. (273)), and confirming these via comparison to wild-type summer honeybees reared *in alvo* via RT-qPCR, we can then investigate the role of QMP specifically.

QMP-exposed bees *in cavea* can then be used to generate samples of the three bee types seen *in cavea*: the individuals which always activate, the bees which never activate, and the bees which only activate in the absence of QMP. The first group collected from QMP-exposed cages, the latter two collected from cages not exposed to QMP.

In order to eliminate any accidental crossover of individuals from these groups large sample sizes e.g. pools of 10 bees per sample, and at least 10 samples; can be used.

3.6.2 Development or plasticity

It is more difficult to investigate the possibility that sexual development has been retarded and then coopted for the mode of reproductive constraint (rather than plastic diapause mechanisms being coopted).

It is possible that the non-sexual development of honeybees which occurs in the first few days post-eclosure can be separated from the sexual development aspect via the withholding of protein in the first e.g. six days after eclosure. As it was shown in section 3.3.3, that a sugar-only diet did not result in increased mortality to the bees, suggesting that ordinary development occurred in a relatively normal manner.

After these six days the rate at which ovaries activate can be compared to the rate at which ovaries activate from eclosure if fed *ad libitum* pollen and fondant. This could then be repeated at day 10, and at day 14.

If the rate of activation is slower, the further away from eclosure, this could be indicative of decreased plasticity with age (as has been observed in other species (199)), and evidence in favour of adult plastic effects, such as diapause. If the rate of activation is the same between day six, 10, and 14, but different between these and day zero, then we see evidence of developmental mechanisms, as initially development would have been slowed via the other non-sexual development. If the rate of activation is the same across all time points, then it is likely an effect of plastic elements, as development is known to correlate in time with protein availability (274).

Chapter 4 – How does *Bombus terrestris* respond to QMP?

4.1 Chapter Introduction

The buff-tailed bumblebee *B. terrestris*, of the Apidae family, exists within the closest extant clade to the eusocial honeybees of the *Apis* genus, separated by some 55 million years (7). This primitively eusocial bumblebee lacks many of the complex social interactions and biological traits that the highly derived *A. mellifera* possesses, but is thought to share a single origin of the evolution of eusociality with the honeybees (7).

Although there are many differences between these species (such as the differences in life cycle and complexity of social organisation (40, 275)) it has been shown that the honeybee QMP is able to repress reproductive activity in *B. terrestris* (209). This makes it an ideal candidate for understanding the role of QMP in the evolution of eusociality in *A. mellifera*.

4.1.1 Bumblebee Biology

B. terrestris audax is a common visitor to British gardens throughout the spring, summer, and autumn (40, 276). Although generally this species hibernates over winter, in urban areas, particularly in the South East, South, and South West of England, it is also able to maintain populations throughout the winter (277, 278), with the limiting factor of its colony lifecycle being access to forageable material, rather than climatic or temperature conditions (278).

4.1.1.1 Lifecycle

The typical colony lifecycle begins when a mated founding queen awakes from an over-wintering diapause in spring to found a new colony. During the founding stage she forages pollen and nectar to rear a small number of eggs to adulthood, before settling in to full-time egg-laying and brood care (40, 279).

After the founding stage, the colony switches over to the eusocial stage. The new workers take over responsibility of foraging, also helping with brood rearing and nest development, rearing more workers until the first major developmental shift within this stage: the queen switches from laying eggs destined to become workers; and instead starts laying eggs destined to become gynes (future queens). This continues until the second major developmental shift within the eusocial stage: the queen switches over to laying unfertilised haploid eggs destined to become males, this is called the switch point (279).

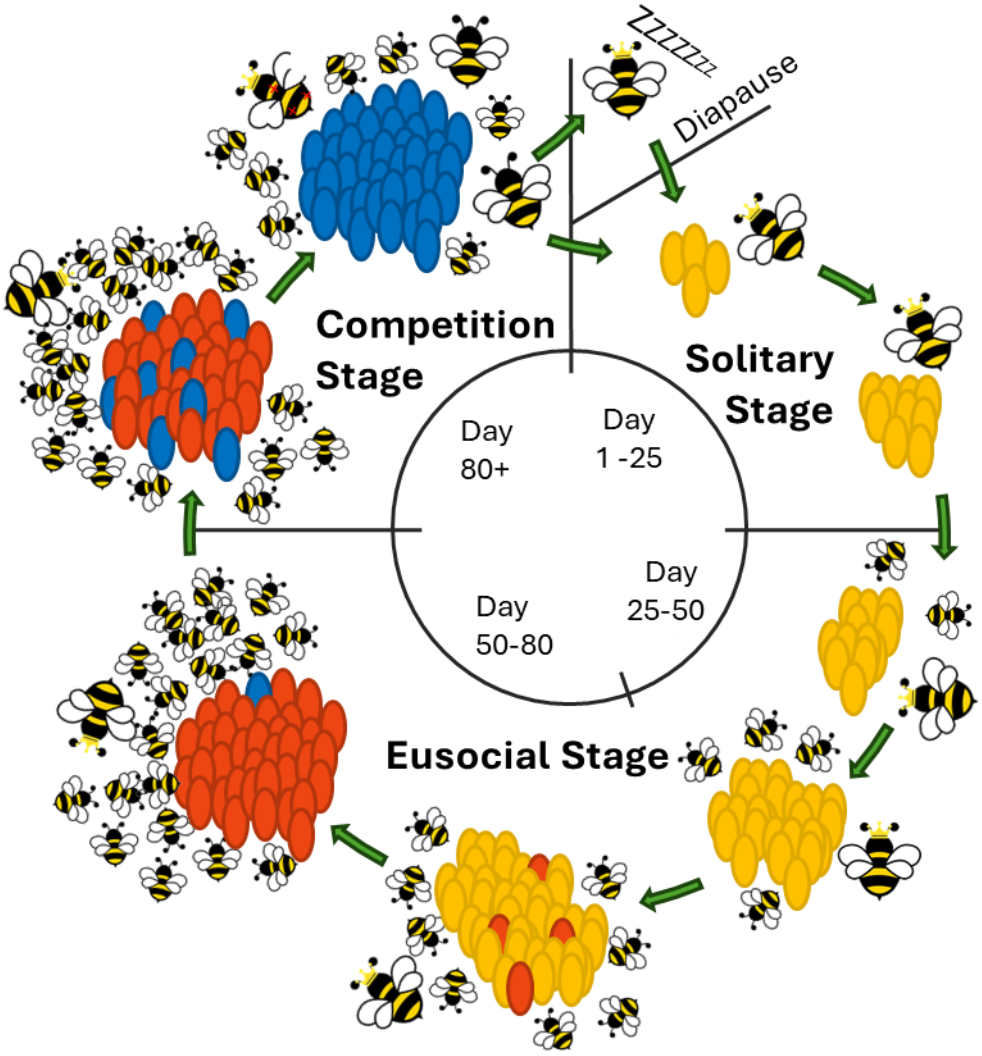


Figure 4.1 – Showing a simple diagram of the bumblebee life cycle. A founding queen produces cells and provisions them by foraging to produce workers (shown as yellow cells), during the solitary stage. As workers first eclose the eusocial stage begins, and worker numbers expand. Towards the middle of the stage the queen switches over to gyne production (red cells). Eventually no worker cells remain, as they have all eclosed. With the first male cell (blue cells) the switch point is reached, shortly followed by the beginning of the competition stage. Shortly after all eggs being to be laid as male, worker competition reaches its maximum, and the queen starts to become more injured as she deals with worker aggression. Adult gyne mate and either immediately found new colonies, or enter into diapause to hibernate through winter first.

Some days later, the competition point occurs, heralding the competition stage where eusociality breaks down. It is no longer in the interest of the workers to look after the male larvae, as their own direct fitness is better served by laying their own eggs. They start to act in

competition with one another and the queen, laying their own eggs and fighting with the queen aggressively.

As gynes emerge, they engage on mating flights before going on either to found a new colony or to find a nesting site in which to diapause over winter, they will often return to the nest after mating, before finding a suitable site.

Eventually, as no new workers are being produced, the colony numbers drastically reduce and the founding queen and workers die. (40)

This lifecycle is detailed graphically in Figure 4.1.

4.1.1.2 Competition between queens and workers and eusociality

Early in the colony lifecycle, before the switch point, the workers do not exhibit any direct competition with the queen. I.e. that their ovaries remain inactive; they engage in no aggressive or combative behaviour with the queen; they do not try to lay their own eggs; or to consume queen-laid eggs (40, 279). A latent degree of competition begins to show towards the end of the gyne period, where the queen beings to spend much of her time guarding the male eggs she has laid, aggressively fighting with workers (notably losing limbs, hair, and wings in the process) (131, 279). This begins the competition phase of the bumblebee lifecycle.

The loss of cooperation between the workers and the queens at this point, signals the end of the eusocial structure of the colony.

B. terrestris bumblebees are considered primitively eusocial, with some of the morphological changes that are associated with eusocial caste-separation (such as significant size difference, but the same number of ovarioles per ovary (40)).

Although it was recently shown that the spermatheca of *B. terrestris* (among other bumblebee species) are able to be functional in a way previously assumed not to be the case. In this paper the workers were artificially inseminated, and also mated manually to males and offspring measured, as well as colony formation. (280)

Therefore, despite forming primitive eusocial colonies with reproductive constraint and a reproductive division of labour, the nature of reproductive constraint and the reproductive division of labour in this species is shallow and short-lived.

4.1.1.3 Pheromones and reproductive constraint

How this order is maintained before the competition point is not very well understood, nor are the precise conditions which produce the advancing of the colony cycle from founding to gyne to competition phases.

It is known in the closely related species *B. impatiens* (<45 million years diverged (7)) that the physical presence of the queen is necessary for workers not to engage in their own egg-production and egg-laying, and that if she is physically separated from the workers, the workers' ovaries start to activate, and they begin to engage in queenless behaviours (90, 281).

Work by Van Oystaeyen et al. (46), Holman (88), and Princen et al. (47) has indicated the role of pheromones in the maintenance of eusocial structure in this species, in the manner of reproductive constraint seen in other hymenopteran insects, especially the honeybees (46, 88, 209). However, the conclusions drawn by the former two papers are disputed in the literature for methodological reasons, particularly by the Amsalem group (282).

In particular, Van Oystaeyen (46) utilised a form of Hess score, based on Duchateau and Velthius' classification of ovary activation (131), whereby ovaries were categorised into inactive, active, and regressed, the latter of which is not shown to be linked to reproductive constraint as it was used in this paper, i.e. it was used as a proxy for reproductive control, despite its presence in 70% of laying bumblebee workers and most queens (131). Holman (88) used "number of developing oocytes present in the ovaries", though with no clear evidence of what this means.

Additionally, Holman's paper uses estimation to categorise the workers into sizes such as "small", "medium", and "large", for which no metric or justification is given, which lacks the adequate rigour of scientific investigation.

In both of these papers, no effort was made to account for prior exposure to queen pheromone, and therefore possible learned behaviours, particularly important as this species is known for its high learning ability (212).

Amsalem et al. (282) utilised a more sterile *in cavea* setup in a different bumblebee species *B. impatiens*, which is not without its flaws. Groups of 3 workers from young colonies were exposed to queen pheromone for 10-13 days before dissection. Ovaries were measured for activation via length of the terminal oocyte in three ovarioles, alongside whether the ovary contained a resorbed oocyte. The cumulative number of eggs laid in each cage was also recorded. The use of small group sizes in this set of experiments could potentially obfuscate

much of the findings by virtue of not adequately mimicking a natural environment. In any case, it is clear that there is still dispute on the role of queen pheromones in this species.

The role of diapause as a potential mechanism of action for the implementation of reproductive constraint has been suggested for other species (103), and the existence of reproductive diapause, at least in the queens of this species, suggest that this might hold true in this species too. Similarly, the role of stress as a factor in reproductive constraint is only starting to be investigated, and very little is known in *B. terrestris*. In *D. melanogaster*, QMP is suggested to work via imitating starvation pathways (183), and that therefore, the mechanism by which QMP is able to produce reproductive repression in this species, would be closely linked to reproductive senescence via stress-related pathways.

Whether the mechanism of action of pheromonally-mediated reproductive repression in *B. terrestris* is as a result of forceful reproductive constraint, against the interest of the workers, and in the interest of the queen (as has been suggested), is unclear. It is also unclear whether this reproductive repression is mediating diapause, or senescence pathways, as it seems to be acting in the fruit fly (see Chapter 6).

4.1.2 Bumblebees as a model

The reported sensitivity of *B. terrestris* worker reproduction to pheromones, particularly honeybee QMP, raises the possibility of this organism being able to be used as a model for understanding the evolution of QMP, and queen pheromones more generally, and their importance in the evolution of eusociality within this clade. Particularly due to the relatively close relationship between the two genera.

The fact that QMP has been shown to have a greater effect on *B. terrestris* worker ovary activation than its proposed native queen pheromone nC25 (209), implies that the widespread activity of QMP is also very potent in terms of its ability to mediate reproductive constraint. (Although, as mentioned in previous sections the activity of QMP is not universal, such as in *V. vulgaris* which is not repressed by QMP (133))

Investigating the mechanism of action of QMP in this species gives us insight into the evolution of QMP in the honeybee. How similar the pathways of QMP-mediated ovary repression are, and how conserved the mechanism of action of QMP is between these two species shows us the evolutionary timeline of the evolution of this pheromone.

B. terrestris bumblebees are particularly useful in this regard as they can be obtained in relatively large numbers throughout the year due to their use as a pollinator in greenhouse

settings agriculturally (208). This allows research to continue throughout the autumn, winter, and spring, when *A. mellifera* work is halted due to their lifecycle.

Bumblebees have been shown to be responsive to experimental setups *in alvo*, due to the small size of their colonies and simple biological requirements. *In cavea* experiments have also been undertaken in this species, but always with some degree of native colony reproduction (such as the presence of comb and brood present from the colony of origin) e.g. (283).

The development of an *in cavea* model, which does not simply replicate a colony as a microcolony for experiments, would provide a sterile environment in which very specific pheromonal, stress, abiotic, or biotic factors could be analysed without many of the confounding variables that normally obfuscate conclusions from *in alvo* experiments, and which are still present in microcolonies.

This *in cavea* model could then be used to investigate the mechanism of action of QMP in this species, as well as the mechanism of action of its own native nC25. Comparisons could then be made to the nature of QMP activity in honeybees, with the evolutionary inferences which could then be drawn.

4.1.3 Research Questions

The research in this chapter will attempt to answer the question, can we establish an effective *in cavea* model, via which we can investigate how QMP is able to bring about reproductive repression of bumblebee workers?:

- Can we establish an *in cavea* model in the lab context, allowing for controlled exposure of bumblebee workers to specific pheromones?
- Using this model can we reproduce published data showing the repressive effect of nC25 and QMP on worker reproduction in *B. terrestris*?
- Once demonstrated as reproducible, are the internal mechanisms by which nC25 and QMP bring about this repression the same as one another, and are they different to those demonstrated in honeybees for QMP?

4.2 Chapter Methods

4.2.1 Bumblebee Colony Rearing

See section 2.3.2.

4.2.2 *In cavea* bumblebee experiments

Different methodologies were utilised for *in cavea* experiments throughout this chapter, summarised presently, however in all cases metal, autoclavable cages (10 cm x 9 cm x 5.5 cm steel with removable glass front and air holes, www.small-life.co.uk) were used as the container, and experiments carried out at 27°C, humidity was kept at 70% ±10%.

Additionally, when moving bees into cages, five bees from the same colony were placed into a 50 ml centrifuge tube and emptied into a hole in the top of the cage. The bees were not anaesthetised and so this was done under pure red light in order to prevent flight (284). Bees from different colonies were not mixed into cages, and so the colony-of-origin (each colony was labelled upon arrival with a letter) was recorded for each cage. Cages were systematically prepared such that an even number of each colony-of-origin was represented in each treatment group, in an attempt to mitigate colony-of-origin effects on treatment.

To optimise *in cavea* experiments a number of parameters were empirically tested:

- Cage laid vertically or horizontally (Figure 4.4), to determine whether space requirements were important
- Either 12h:12h Light:Dark cycle, or completely dark except exposure to red light, to investigate the stress effect of light exposure on mortality
- With 5, 10, or 15 bees per cage, as numbers of bees are linked to social hierarchies (285)
- Fed liquid either via top or side of cage, to determine cage setup for ease of access
- Solid food either CBF (see section 2.2.3.1.1), whole pollen grains (LiveMoor), ground pollen grains (LiveMoor, ground in a coffee grinder); to determine the most biologically relevant feeding regime with high survival
- Liquid food either water, 70% w/v sucrose in water, BioGluc (Agralan), to determine the most biologically relevant feeding regime with high survival

By the end of the methodological development aspect of this chapter, the optimised conditions were as follows:

- Vertical orientation

- Kept in dark except red light for working
- 10 bees per cage
- Fed liquid via side of cage
- Solid food as whole pollen pellets *ad libitum* refreshed every two days
- Liquid food as Biogluc refreshed as necessary

Additionally, the pheromone treatments applied differed in concentration, and these are given where appropriate according to pheromone concentrations given in section 2.2.1.

BioGluc and pollen was supplied *ad libitum*, treatment was administered daily, and the length of the experiments ran either seven days, or 10 detailed in each relevant section.

4.2.2.1 Pheromone exposures

The pheromones QMP and nC25 were used *in cavea*. Each experiment has its concentrations defined throughout the chapter in the relevant sections. Pheromone concentrations are measured in Qe.

QMP is dissolved in ethanol, and nC25 in pentane, requiring direct comparison between pheromones to include the solvent control of the other. E.g. QMP + pentane, and nC25 and ethanol, with a negative control of both solvents.

For more information see section 2.2.1.

4.2.2.2 Starvation and partial food experiments

The feeding regimen for the experiments laid out in sections 4.3.1.8 and 4.3.2.3.4, differed from other experiments in that the food used was mixed with methyl cellulose, a biologically inert substance used to bulk the protein source to reduce its quality (286).

In section 4.3.1.8 this was characterised as Low, Medium, and High, the mixtures of which are given in Table 9.

The experiment in section 4.3.2.3.4 utilised the Medium quality mixture.

Table 9 – Showing the proportions of methylcellulose and ground pollen used for the starvation and partial starvation experiments

Mixture	% methylcellulose	% ground pollen
Low	90	10
Medium	50	50
High	10	90

4.2.3 Bumblebee Ovary Activation Measuring Methods

4.2.3.1 Main Hess score system used throughout

A modified Hess score was constructed based on Duchateau et al. (131) and Geva et al. (287) which in turn was based on the scoring system developed by Hess et al. (130).

The *B. terrestris* Hess score differs from that used in *A. mellifera* as defined in section 2.6.1.1. This is because bumblebee ovaries intrinsically display a greater degree of ovary activity than honeybee ovaries. The simple Hess score used throughout this chapter is defined as in Figure 4.2

4.2.3.2 More precise Hess score system

A more precise modified Hess score was also developed, in which there exists greater precision between different qualitative categories.

This followed the following scheme:

0 = no discernible oocytes/nurse cells

1 = discernible but small oocyte, smaller than nurse cells, spherical.

2 = oocyte still smaller than nurse cells but elongated and flat topped

3 = oocyte as big as or larger than nurse cells

4 = nurse cells degenerating

5 = fully mature oocyte, no nurse cells present

6 = reabsorbed oocyte / two mature oocytes present in different ovarioles

7 = two consecutive fully mature oocytes present in one ovariole

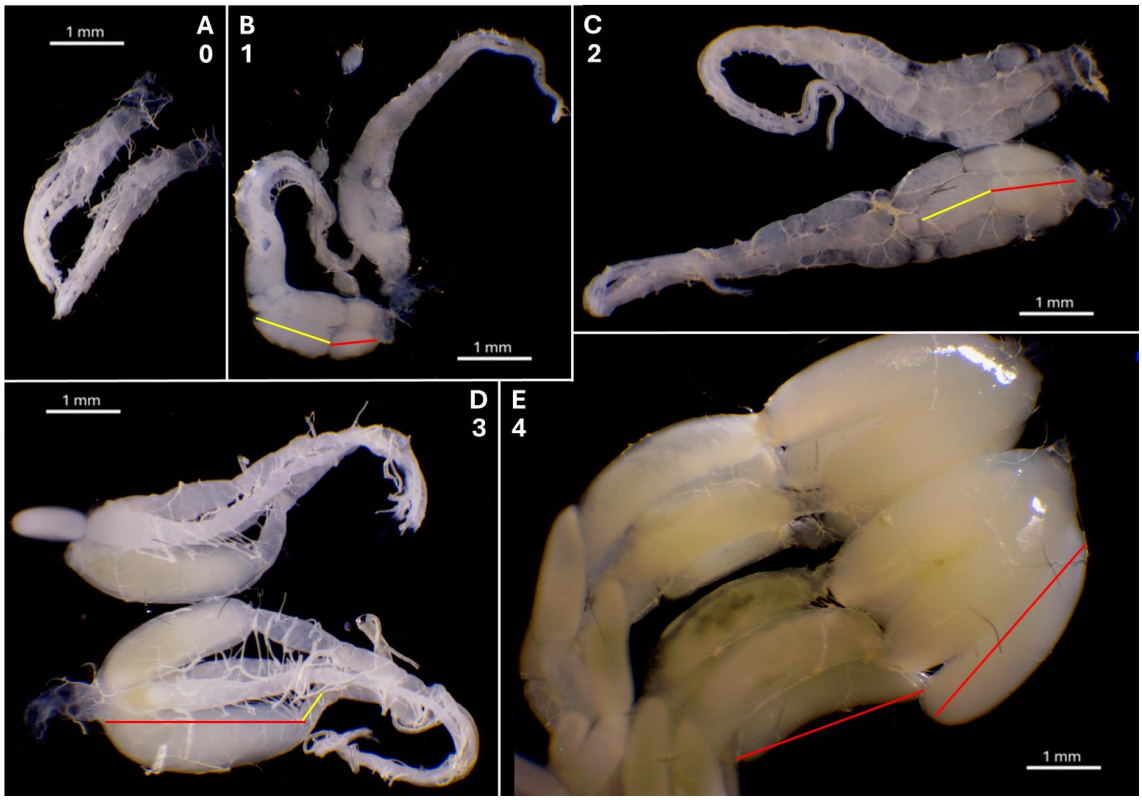


Figure 4.2 – Showing the simple modified Hess score (0-4). Shown in panels A-E respectively, for characterising the development of *B. terrestris* worker ovaries. A shows ovary score 0, defined by no discernible differentiation between nurse cells and oocyte. **B** shows ovary score 1, defined whereby cell types are discernible by presence of yolk in the oocyte, but the oocyte (shown in red), is shorter in length than the nurse cells (shown in yellow). **C** shows ovary score 2, defined whereby cell types are discernible by presence of yolk in oocyte, and the oocyte (shown in red) is the same length as, or longer than the nurse cells (shown in yellow). **D** shows ovary score 3, defined by the atrophying of nurse cells (shown in yellow), and almost completely developed oocyte (shown in red). **E** shows ovary score 4, defined by the presence of a completely developed oocyte (shown in red), and lack any visible nurse cells between the oocyte and the adjacent oocyte (also shown in red). All panels are to the same scale with scale bars as 1 mm.

4.2.3.3 Quantitative ovary measurements

Images of the extracted bumblebee ovaries, submerged in PBS were taken at 6.75x magnification, using a Leica binocular dissection microscope and a GXCam microscope camera using the GXCapture software (GT Vision, UK).

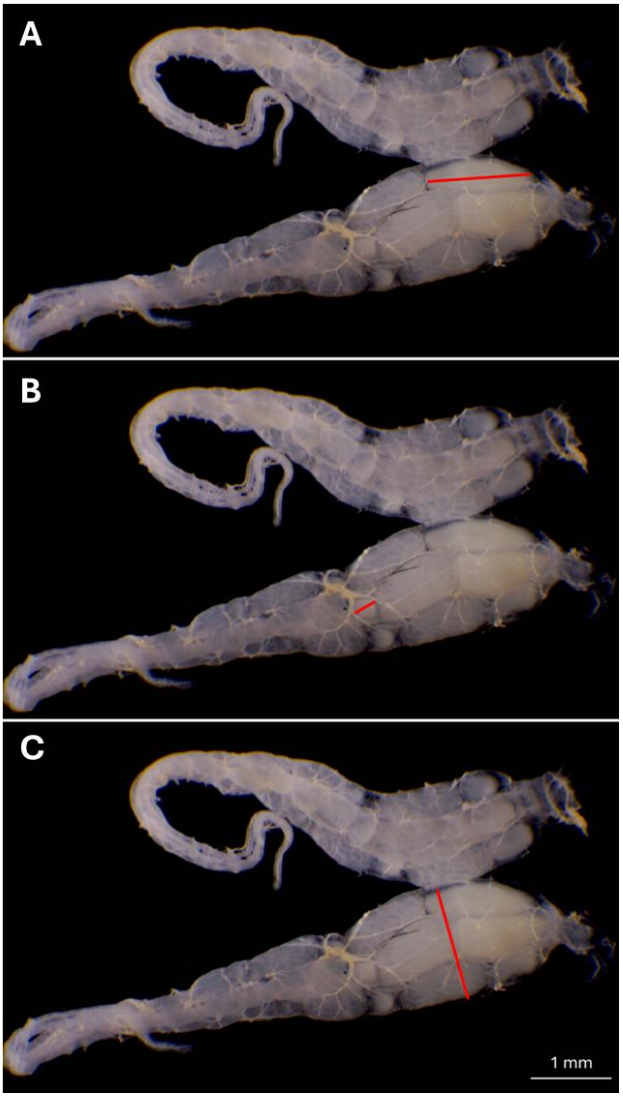


Figure 4.3 – Showing the different methods of quantitatively measuring the same *B. terrestris* ovary for measuring its ovary development. **A** shows the measurement of the terminal oocyte. **B** shows the measurement of the proximal oocyte. **C** shows the measurement of ovary width. All measurements made are shown in red. All images are to the same scale, scale bar is shown as 1 mm.

4.2.3.3.1 Length of terminal oocyte

Images of the extracted ovaries were processed in ImageJ (Fiji, v. 2.1.0/1.53c), where the length of the longest and most fully developed oocyte at the terminal end of the ovariole was measured in pixel length, as demonstrated in red in Figure 4.3**A**. The pixel measurement was then converted to millimetres using an image of a ruler taken at the same magnification as a reference.

4.2.3.3.2 Length of proximal oocyte

Images of the extracted ovaries were processed in ImageJ (Fiji), where the length of the longest oocyte proximal to the terminal oocyte was measured in pixel length, as demonstrated in red in Figure 4.3**B**. The pixel measurement was then converted to millimetres using an image of a ruler taken at the same magnification as a reference.

4.2.3.3.3 Ovary width

Images of the extracted ovaries were processed in ImageJ (Fiji), where the width of the ovary at the greatest width was measured in pixel length, as demonstrated in red in Figure 4.3**C**. The pixel measurement was then converted to millimetres using an image of a ruler taken at the same magnification as a reference.

4.3 Results

B. terrestris represent an extant relative of *A. mellifera* within the Apidae family, and although separated by ~55million years, are thought to share a common origin of eusociality (7), and have been shown to be able to be reproductively constrained with the honeybee QMP (209). They are also able to be reared within a lab context, and so could provide a powerful tool for investigating evolutionary questions regarding the phylogenetically widespread activity of QMP.

In order to use this organism to investigate the mechanism of action of QMP and draw inferences about the evolution of QMP and its impact on eusociality, we first had to establish the *in cavea* method used in Chapter 3 for *A. mellifera* workers with *B. terrestris* workers.

4.3.1 Methodological Development

The first several experiments were to investigate different methods to optimise maintaining the *in cavea* workers with acceptable mortality rates.

4.3.1.1 Cage orientation has no effect on survival of *B. terrestris* workers

It was observed very quickly that, while honeybee workers were able to climb the side of the metal cages used to carry out *in cavea* experiments, the bumblebee workers were unable to do so. As a result, a test checking whether the orientation of the cage might produce lower mortality rates was undertaken.

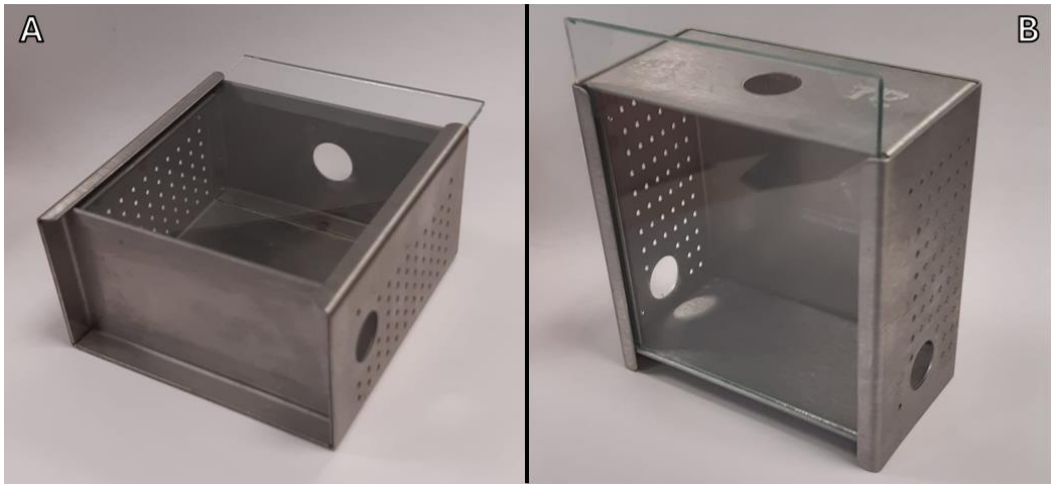


Figure 4.4 – Showing the two possible orientations of a cage. **A** shows the horizontal orientation where the “back” of the cage is laid flat with the surface of the table. **B** shows the vertical orientation, where the “foot” of the cage is laid flat with the surface of the table.

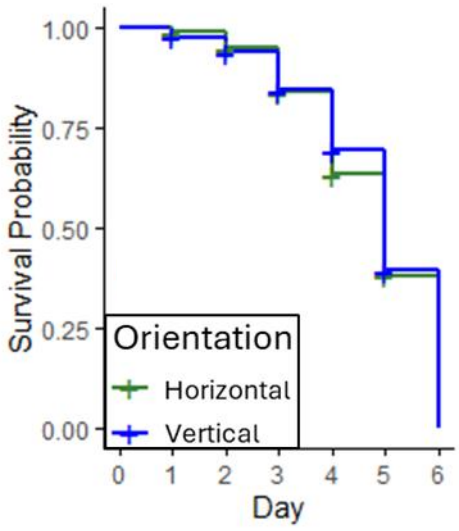


Figure 4.5 – Showing a Kaplan-Meier survival curve presenting the effect of cage orientation on survival probability of *B. terrestris* workers. The y-axis shows survival probability. The x-axis shows day of experiment. The colours represent cage orientation, where the vertical conformation has the longest length of the cage as vertical, and the horizontal has the longest length of the cage as horizontally placed. The graph shows no difference between orientations, but both orientations in this assay demonstrated 100% mortality by day 6.

The horizontal orientation refers to a cage which lies on its back. As shown in Figure 4.4**A** while the vertical orientation refers to a cage standing upright, as shown in **B**.

As can be seen in Figure 4.5, there is no difference in mortality between conformations (χ^2=0.0467, df = 1, P=0.8289). It must be noted that mortality was 100% in all cages tested by the end of the experiment.

As there was no difference in rate of death, the vertical conformation was chosen for practical reasons: it was better able to be stacked in the space available; it provided marginally better access to the feeders (as the space between the "bottom" of the cage and the feeding holes is less when the cage is oriented vertically); and the glass cover plates were less likely to be accidentally dislodged than in the horizontal orientation.

4.3.1.2 *B. terrestris* workers require liquid sugar in an *in cavea* setup

The different potential food types formed the basis of the next series of experiments. While the bees are being reared in their original colonies, they are fed on a diet of whole grain pollen collected from honeybee colonies (LiveMoor), and a nutritionally complete commercially available sugar liquid BioGluc (Agralan).

Initially however we needed to test if the same setup being used for the honeybees at the time was able to produce similar results in the bumblebee cages, in order to facilitate direct comparisons between the two species. This setup was using CBF food as a solid food source, and water as a liquid food (with the assumption that the sugar needs were satisfied by the CBF). This setup was different to that which the bees were being reared on in the colony setting, and so it was investigated whether CBF was able to be nutritionally viable without an additional sugar supply.

As can be seen in Figure 4.6, there is a marked statistically significant decrease in mortality when the bees are fed sugar in addition to CBF (χ^2=78.221, df = 1, P=<0.0001). Indeed, when only given water, the bees showed 100% mortality by the end of day 3. This possibly indicates that the worker bumblebees do not consume CBF as a food source in the way that honeybees do.

Therefore, it was deemed vital that the bumblebees had access to a source of liquid sugar during the course of an *in cavea* experiment.

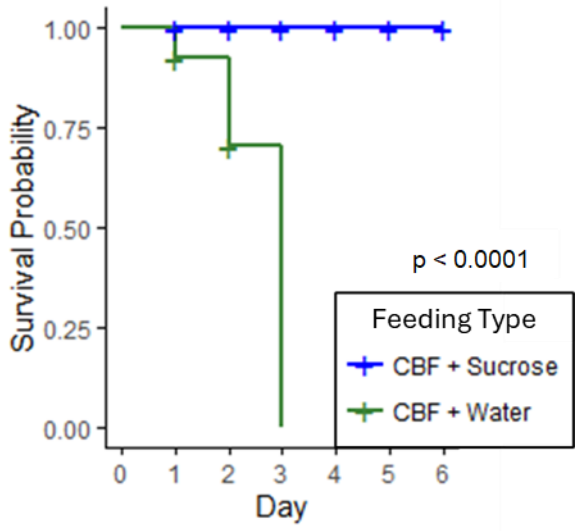


Figure 4.6 – Showing a Kaplan-Meier survival curve of *B. terrestris* workers fed with or without sugar solution. The y-axis shows survival probability and the x-axis shows day of experiment. The colours show different feeding types with one group fed CBF as a solid food and sucrose as a liquid food, while the other is only provided CBF with water as a liquid. Probability was calculated using a Log rank test. The graph shows a clear difference in survival rates between bees fed sugar solution or water, with mortality in the water group reach 100% by day 3.

4.3.1.3 *B. terrestris* survival in lit conditions is markedly lower than in total dark

The incubator setup used to rear the honeybees includes access to light when the incubators are opened and closed, and it was noted that when the incubators are opened and closed more, there was higher mortality of the bees in the following days.

It was hypothesised that the stress induced by the exposure of light was causing an increase in mortality. This was tested via exposing one group of bees to a 12:12 light dark cycle, and the other total dark, to see the impact on mortality.

As can be seen in Figure 4.7, exposure to light produced a significantly increased probability of mortality for the bumblebee workers (χ^2=134.12, df = 1, P=<0.0001).

As a result it was deemed vitally important not to expose the bees to any light during the course of the experiments, and a separate controlled-temperature room was setup to accommodate this.

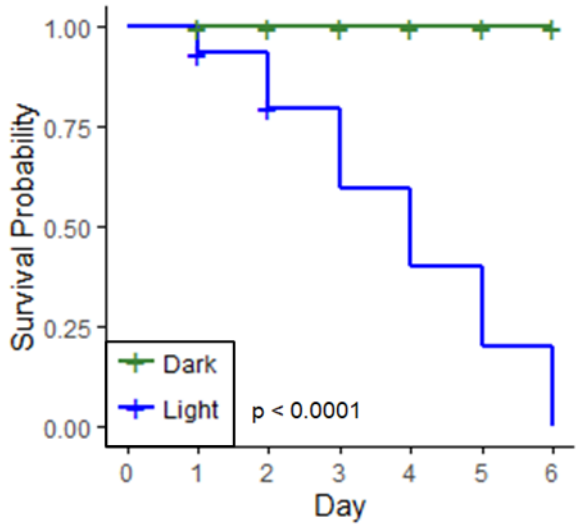


Figure 4.7 – Showing a Kaplan-Meier survival curve of *B. terrestris* workers in light and dark conditions. The y-axis shows survival probability and the x-axis shows day of experiment. The colours represent light and dark conditions, where dark is kept fully in darkness for 24h a day, and Light is in a dark light cycle of 12h-12h. The probability was calculated using a Log rank test. The graph shows clear high mortality of bees exposed to light with 100% mortality within 6 days.

4.3.1.4 *B. terrestris* worker cages must be fed liquid food from the side not the top

The different feeding holes through which the bees could be fed liquid food was tested. This was because of the same observations made in section 4.3.1.1, where it was observed that the bumblebee workers seem unable to climb the walls of the cage. If the bees had reasonable

survival on liquid feeding from the top of the cage, then it would allow two different feeding holes for application of food from the side of the cage. As a result, feeding via top or side was investigated for their mortality rates.

As can be seen from Figure 4.8, there was a significant difference in survival probability between the two methods (χ^2=768.31, df = 1, P=<0.0001).

This marked decrease in survival of top feeding relative to side feeding conclusively shows that the bees must be fed from the side, where they are likely better able to access the liquid food.

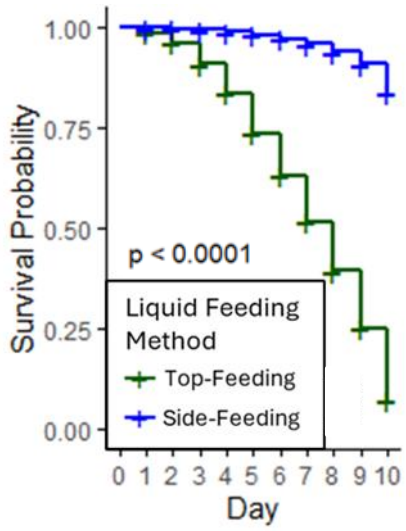


Figure 4.8 – Showing a Kaplan-Meier survival curve of different methods of giving sugar-liquid to *B. terrestris* workers in cages. The y-axis shows survival probability. The x-axis shows time of experiment in days. The different colours show the two different sugar-liquid feeding methods: fed via the top of the cage or via the side. The probability was calculated using a Log rank test. The graph shows clear difference in survival between the two feeding methods.

4.3.1.5 The number of bees per cage has little effect on survivability or on ovary activity

The larger the number of bees per cage the greater opportunity for worker-worker aggression, and the formation of reproductive hierarchies, which act as potentially confounding variables when investigating the role of queen pheromones. A balance must be struck, between enough bees for realistic behaviour, but not so many as to bring about reproductive repression via behavioural means which could obfuscate the pheromonal response. (283)

As a result, an experiment was undertaken to investigate both ovary activity and mortality in bees from cages with different numbers of bees. The results of which can be seen in Figure 4.9 and Figure 4.10.

In Figure 4.10 we see there is no statistically significant difference between cages of different numbers of bees (F = 2.9949, df = 2, P=0.0518), though there is a non-statistically significant trend towards smaller ovaries in cages with larger numbers of bees with much higher variance (10:15 = P=0.0617), individual pairwise test results detailed in Table 37, in appendices.

A power analysis showed the n-value needed to determine significance was at roughly n=110, and so the dataset may simply have been slightly too small to have demonstrated a significant effect size.

Similarly, there was no difference in mortality between the different test groups, as can be seen in Figure 4.9.

These results demonstrate that there may be a problem with placing as many as 15 bees per cage, due to marginal suppressive effects of this number of bees in a given cage. Dominance hierarchies have been shown to occur with as few as four bees per cage (264). It has also been shown that the larger numbers of bees present in a group, the greater the aggression between workers (283).

As a result, the number of bees per cage going forward from this point was chosen to be 10, as a compromise between ensuring a biologically relevant social environment, with enough variation, with enough sample size, and without obfuscating pheromone effects with aggressive behaviours.

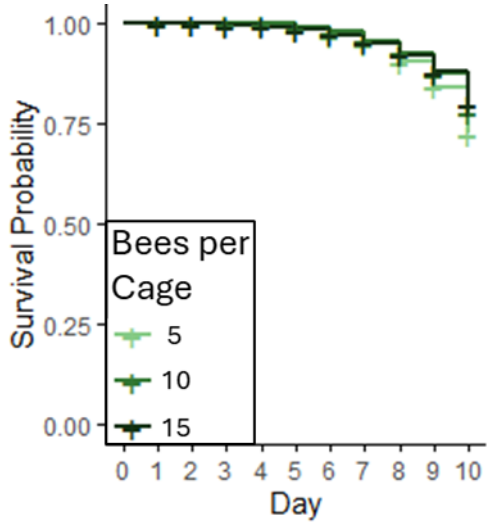


Figure 4.9 – Showing a Kaplan-Meier survival curve of numbers of bees per cage in *B. terrestris* workers. The y-axis shows survival probability, and the x-axis shows day of experiment. The different colours show different numbers of bees per cage. The graph shows no difference in survival according to numbers of bees in cage.

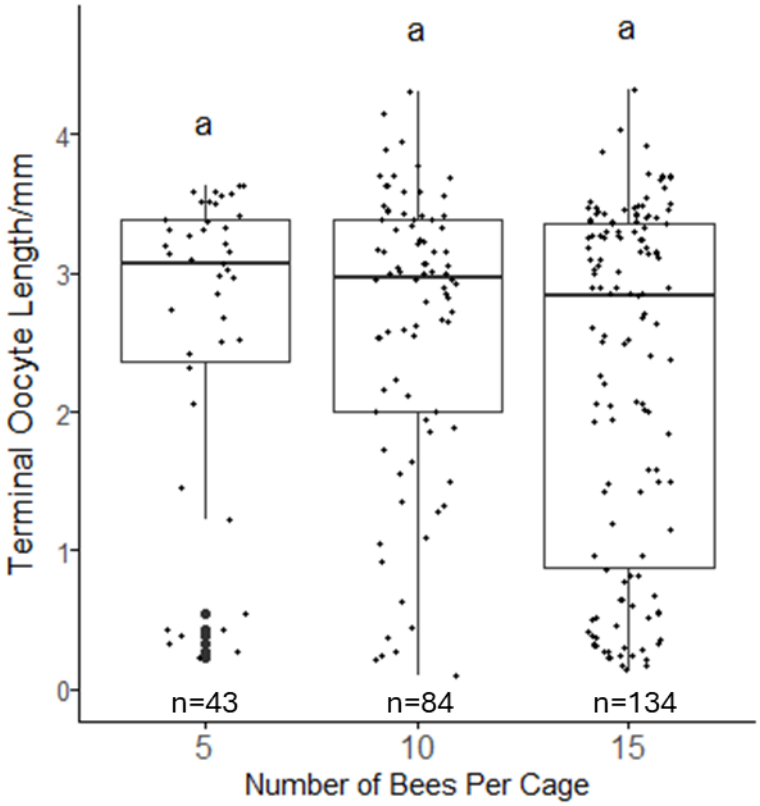


Figure 4.10 – Showing the effect of numbers of bees per cage on the ovary activity of the bees in those cages, as measured by the length of the largest terminal oocyte. The y-axis shows length of terminal oocyte in mm, and the x-axis shows the number of bees per cage. Statistical significance is given as letters ($P<0.05$), calculated via GLMM and post-hoc Tukey pairwise test. The graph shows no statistically significant effect on ovary activity by numbers of bees per cage.

4.3.1.6 Hess score is the most appropriate method of scoring ovary development

There are a number of different ways of measuring ovary activity in bumblebee workers (46, 88, 209). As with other investigations using this phenotype, the feasibility of each is dependent both on the organism being investigated, but also the laboratory conditions, and the researcher themselves due to observer bias.

As a result, four different methods were used to investigate the ovary activity on a single dataset (i.e. a dataset comprised of using different measurements of the same ovaries) to compare and contrast each.

A modified Hess score, laid out in section 4.2.3.1, was used. In this semi-quantitative classification system, the more developed an ovary the larger the number, as defined by given qualitative descriptions.

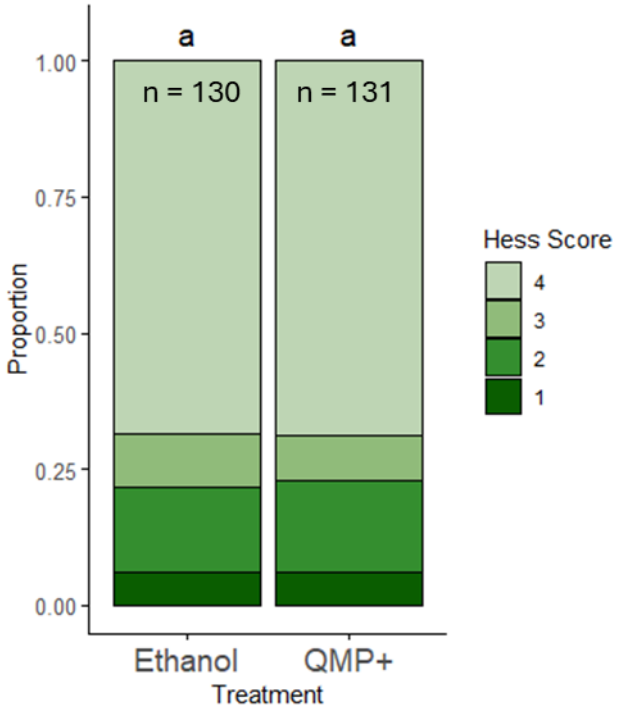


Figure 4.11 – Showing the data from the number of bees per cage experiment aggregated according to treatment in *B. terrestris* workers. The graph shows the effect of treatment on the ovary development measured by modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid). The y-axis shows proportion of each treatment group categorised by modified Hess score. The x-axis shows either ethanol solvent control or honeybee QMP. Significance given as letters ($P<0.05$) calculated via CLMM and post-hoc Tukey pairwise test. The graph shows no effect of treatment on ovary development.

Using the dataset for the previous section (4.3.1.5), where half the bees had been exposed to QMP which has been shown to produce a repressed phenotype in bumblebees when measuring both ovary activity and eggs laid by workers (288); a modified Hess score produced no significant difference in ovary activity between treatments, as can be seen in Figure 4.11 ($\chi^2=0.3311$, df = 1, $P=0.565$). Indeed, this model included number of bees per cage and this variable was a far better predictor for difference of ovary activity ($\chi^2=4.3907$, df = 1, $P=0.03614$).

The three other methods for investigating ovary activity involved imaging and measuring different elements of the ovary: its width; length of terminal oocyte; or length of proximal oocyte. The terminal oocyte being the longest oocyte at the terminal end of an ovariole; and the proximal oocyte being the longest oocyte adjacent to the terminal oocyte.

The width of the ovary was chosen as a measurement form, as when an ovary is more developed, often a greater proportion of the ovarioles exhibit greater development individually, which increases the width of the ovary at the widest point. The results for this analysis can be seen in Figure 4.12**A**, where there was no difference in ovary activation between the two treatments (F =0.197, df=1, P=0.6575). Number of bees per cage in this model was not a good predictor of ovary activity (F=1.3649, df= , P=0.2438).

The length of the largest terminal oocyte was chosen as a measurement, as it approximates a modified Hess score (by ranking the ovary development according to the most advanced development of an oocyte in the ovary), but does so in a more quantitative way, allowing for more robust statistical analysis. It is also less subjective, as it is dependent upon measurement, rather than classification. As can be seen in Figure 4.12**C**, there was also no statistically significant difference in ovary activation according to this method (F=0.004, df=1, P=0.9493), however in this mode of measurement, the number of bees per cage was a good predictor of model output (F=4.2264, df=1, P=0.0383).

The length of the proximal oocyte was chosen as a measurement form, as bumblebee workers often exhibit a large base proportion of activated ovaries, and so the measurement of the terminal oocyte often masks the degree of activity in a given cage. This was hypothesised to be corrected by measuring the greatest development of the second oocyte in a given ovary). However, as can be seen in Figure 4.12**B**, this also failed to distinguish a difference between treatments (F=0.0614, df=1, P=0.8045), though in this model the number of bees per cage also did not produce a better fit as a variable (F=2.23, df=1, P=0.1366).

Given that none of the four methods of testing was able to differentiate any difference between treatments, it was reasonably concluded that 0.25Qe of QMP was unable to bring about repression of ovaries *in cavea* in this setup.

The testing method chosen going forward however was based on observations made during data collection. Ovary width was rejected as it included a heavy bias rooted in the size of the bee, as larger bees had larger ovaries regardless of ovary activity. Indeed, in these bees egg size seemed to be proportional to size too, with these bees laying larger eggs.

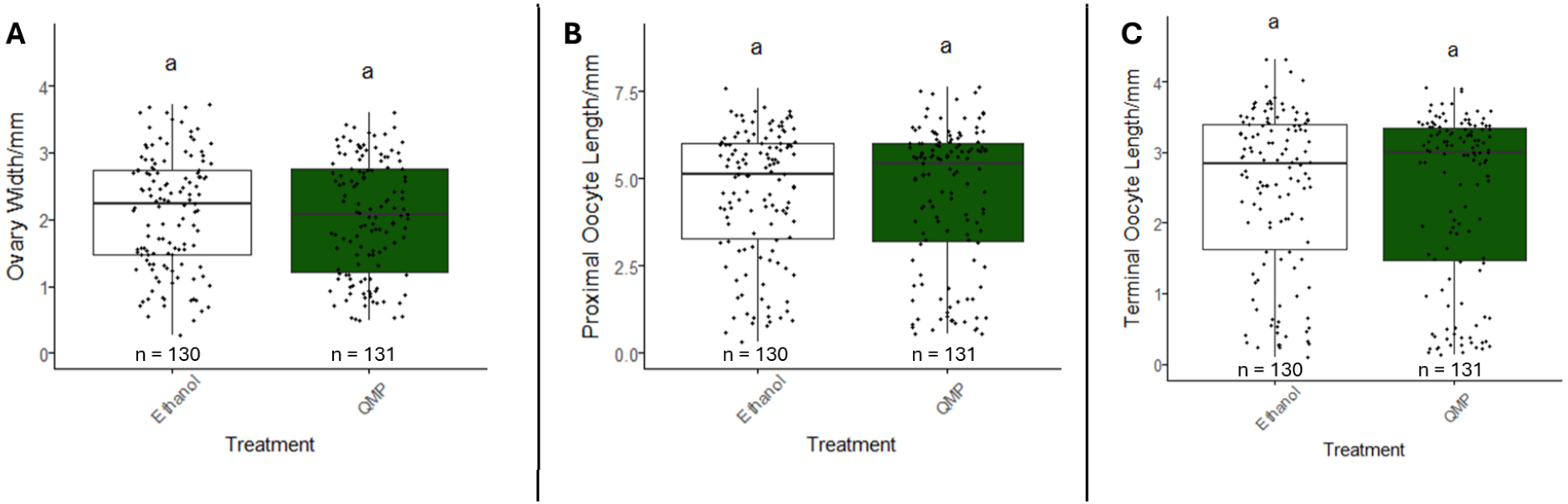


Figure 4.12 – Showing the effect of treatment on ovary activation measured in three different ways in *B. terrestris* workers. In all panels the x-axis shows ethanol solvent-control or QMP treatment. In **A** the y-axis shows ovary activation via measurement of the width of the ovary at the greatest width. In **B** the y-axis shows ovary activation via measurement of the length of the largest oocyte proximal to the terminal oocyte present in the ovary. In **C** the y-axis shows the ovary activation via measurement of the largest terminal oocyte present in the ovary. In all panels significances are given as letters ($P<0.05$) calculated via GLMM and post-hoc Tukey pairwise test , and are independent across panels. In all cases there was no difference between treatments.

Additionally, length of proximal oocyte was rejected based on the difficulty with which measurements could be made in relatively undeveloped ovaries, where the location of the proximal oocyte could not be observed. As a result the full range of biological variation could not be capture with this measurement.

The length of terminal oocyte seemed the most accurate of the three quantitative measurement methods for its ability to discern the ovary activity of the bee, however it too was rejected based on the same criticisms laid out earlier: it fails to adequately differentiate between the higher development categories; but also that it is strongly correlative with bee size, in a similar fashion to that of the other quantitative methods, but especially ovary width.

As a result, the Hess score method was that which was chosen for later experiments. This is also in line with the methods more predominant in the literature (209, 282).

4.3.1.7 There is no difference in mortality or ovary activation between bees fed different protein types

Work carried out throughout the course of this thesis showed that the use of CBF as a food source for honeybees, was less appropriate than the FandP diet (see section 3.3.1).

At the same time this transition was made in honeybees, a more biologically relevant protein feeding regimen was trialled in bumblebees in an attempt to induce QMP-mediated repression of bumblebee worker ovaries as has been published in the literature (209). This was to test the hypothesis that the protein-rich diet the bumblebees were being fed in *in cavea* did not accurately mimic natural feeding conditions. This was theorised to be the case as the mortality in honeybee cages was much higher with CBF feeding than with the more biologically relevant FandP diet.

Additionally, by this point in the timeline of the research, the bumblebees had been switched from sucrose provided *in cavea*, to BioGluc which is both more natural (more closely mimicking plant nectar), but also more consistent with what they are being fed *in alvo*.

The bees were exposed to three different protein feeding regimens: CBF; whole pollen pellets collected from honeybees; or the same pollen pellets ground into a fine powder. All cages were fed these protein types *ad libitum* as well as BioGluc. The bees were also split into two treatment groups of QMP at 0.25Qe/day, and a solvent control.

As can be seen in Figure 4.13 (χ^2=11.501, df = 5, P=0.0423), although treatment and feeding method is a reasonable predictor of ovary activity, there is no statistically significant difference between different protein types, although it appears there is a non-statistically significant

difference between CBF and the two pollen types in the bees exposed to solvent control (P=0.068; individual pairwise comparisons in Table 40 in appendices).

The different protein types also had no difference in effect on the mortality of the bees in these cages, as can be seen in Figure 4.14 (χ^2=5.094, df = 2, P=0.078).

Given that there was no significant difference in mortality between protein types, and that there seemed to be a non-statistically significant difference between CBF and pollen types, it was decided to move forward with pollen as a protein source rather than CBF. As there was no difference between ground or whole pollen, whole pollen was chosen as it requires less processing before application, and represents a more natural manner of pollen exposure for this species.

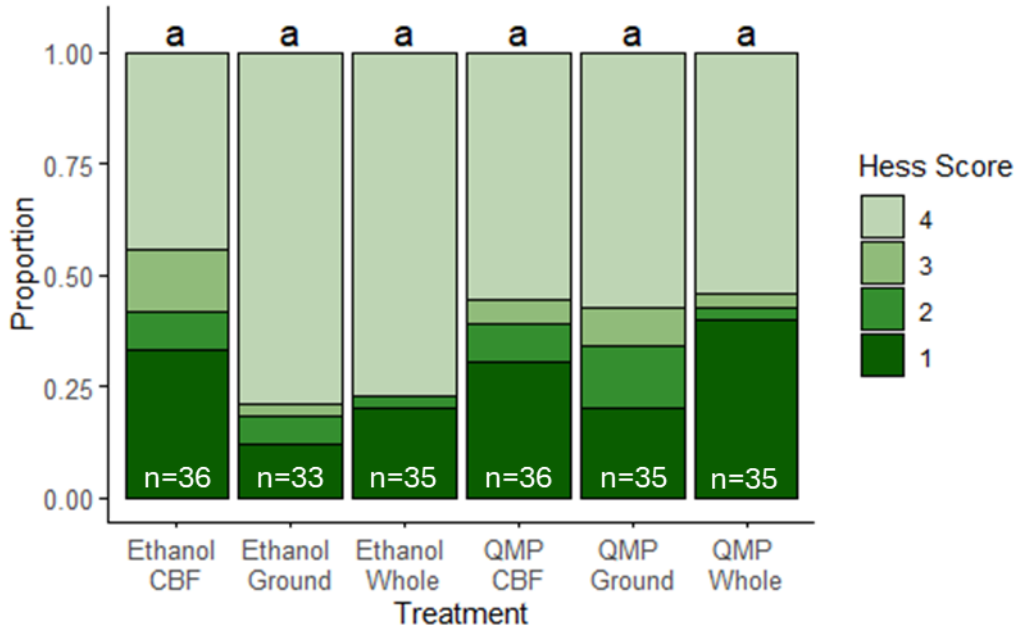


Figure 4.13 – Showing the effect of treatment and protein type on the ovary activation of *B. terrestris* workers via modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid). The y-axis shows proportion of each treatment group categorised by modified Hess score. The x-axis shows treatment of ethanol solvent-control or honeybee QMP, as well as one of three different protein feeding methods: CBF, ground pollen as a powder, or whole pollen pellets. Significances given as letters (P<0.05), calculated via CLMM and post-hoc Tukey pairwise tests. The graph shows no significant difference between treatment groups.

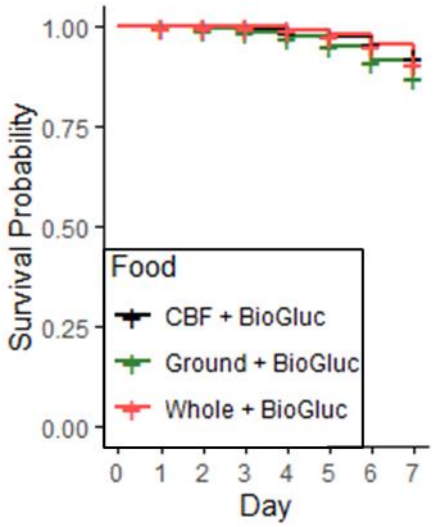


Figure 4.14 – Showing a Kaplan-Meier survival graph of different protein feeding regimens' effects on worker survival in cages of *B. terrestris* workers. The y-axis shows survival probability and the x-axis shows day length of experiment. The different colours show different protein regimens. The graph shows no difference in survival across different protein feeding regimens.

4.3.1.8 Reducing the quality of protein source possibly reduces ovary activity

It has been suggested that in *A. mellifera* the method by which QMP is able to produce ovary repression is by taking advantage of stress pathways (103). As a result, difficulty in reproducing previous findings showing the ability of QMP to induce ovary repression in bumblebee workers, led to the hypothesis that this may be due to the lack of aggregated stress that a more naturally maintained colony would receive.

As a way of simulating stress, we fed different cages of bees with decreasing proportions of pollen, by mixing it with methylcellulose, a nutritionally inert substance (286).

The results of this experiment can be seen in Figure 4.15 (χ^2=3.975, df = 2, P=0.137). In this graph there are no statistically significant interactions (results of individual pairwise comparisons in Table 42 in appendices), and in this model "Food quality" did not show as a reasonable predictor of effect, likely caused by overly small sample sizes. As a result of this, no concrete conclusions can be drawn, and greater sample sizes have to be generated in order to clarify this model.

Although sample sizes are low due to limited availability of bees during this experiment, in comparison to the normal levels of ovary activity present across all investigations, the levels displayed in this graph are of a much lower activity than normally seen.

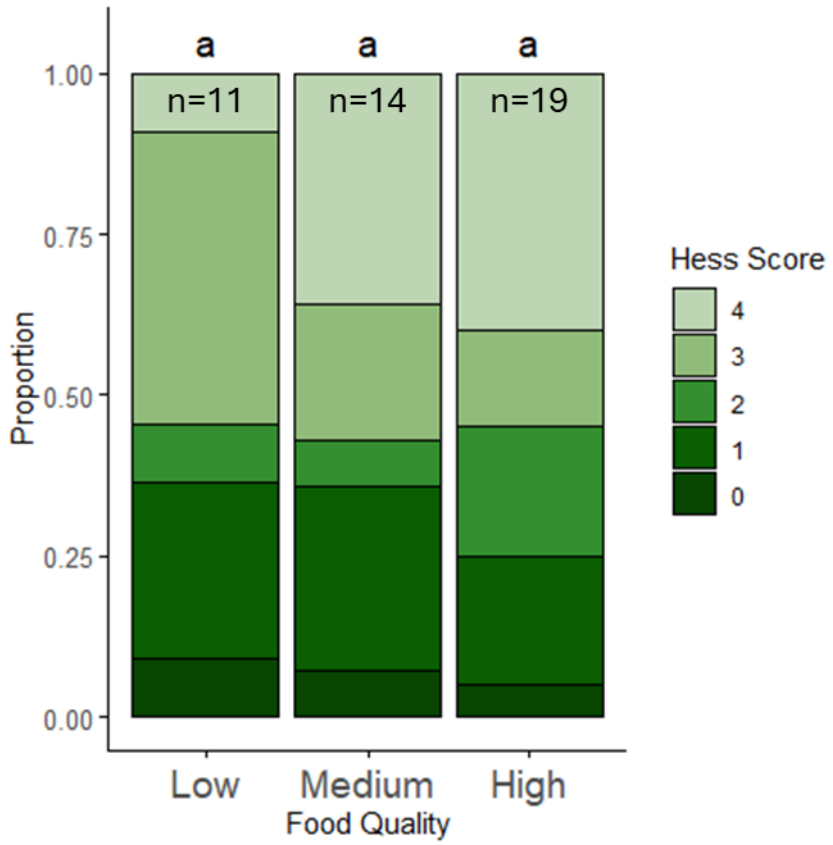


Figure 4.15 – Showing the effect of different proportions of protein mixed with methyl cellulose on ovary development in *B. terrestris* workers as measured by a modified Hess score. (0 = no possible distinction between oocyte and nurse cells; 1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid). The y-axis shows proportion of bees at a given Hess score and the x-axis shows different food groups, with Low representing 9:1 methylcellulose:pollen; Medium representing 1:1 methylcellulose:pollen; and high representing 1:9 methylcellulose:pollen. Significances are given as letters ($P<0.05$) calculated via CLMM and post-hoc Tukey pairwise tests.

4.3.2 *B. terrestris* response to pheromones

In order to investigate the mechanism of action of pheromones in *B. terrestris*, we first had to demonstrate the ability to repress the ovary activity of bumblebee workers in our *in cavea* setup. Given the widely acting nature of QMP as a repressor of reproduction in the insects, the emphasis was placed on this pheromone, however the posited native queen pheromone pentacosane was also investigated.

4.3.2.1 *B. terrestris* ovaries activate to a greater degree in the presence of pentacosane

It has previously been shown that *B. terrestris* workers have their ovaries repressed in the presence of the CHC pentacosane (nC25), which, as a result has been classified as a queen pheromone in this organism.

The first step in investigating the mechanism of action of this pheromone, in order to compare it to QMP, is by reproducing its ability to repress reproduction in *B. terrestris* workers.

However, as can be seen in Figure 4.17**A**, we were unable to reproduce this effect and pentacosane was unable to repress ovary activity in workers when compared to a pentane solvent-control. Indeed, there was a significant increase in ovary activation under these treatment conditions (χ^2=4.5925, df = 1, P=0.0321).

In this model the length of the forewing was also used as a proxy for size of bee, and this was also able to significantly predict ovary activity (F = 9.921, df=3, P=0.0485), shown in Figure 4.17**B**, however when a post-hoc pairwise test was applied to this model, reasonably conservative adjustment for multiple test comparisons (Tukey) pushed the probabilities into non-significance (P=0.095, results of individual pairwise comparisons in Table 45, in appendices). It is possible that this represents a type II error as a result.

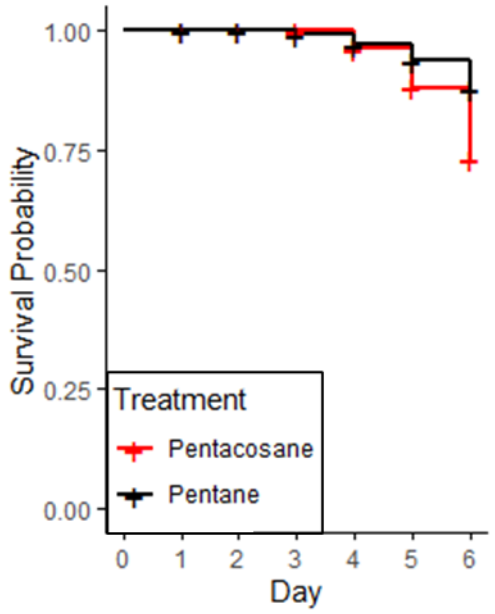


Figure 4.16 – Showing a Kaplan-Meier survival curve of *B. terrestris* workers exposed to pentacosane or solvent control. The y-axis shows survival probability and the x-axis day of experiment. The colours show treatment. There is no significant difference in survival between treatments.

There was also no effect on mortality as a result of nC25 treatment, as shown in Figure 4.16 (χ^2=2.569, df = 1, P=0.109).

This experiment was carried early in the process of determining the optimal conditions for *in cavea* studies, and so the results might simply reflect sub-optimal experimental conditions. The samples sizes were relatively low, and although statistically significant, at this point in the experimental process, colony-of-origin was not being recorded, and this might account for the difference in ovary activity. After this point, Biogluc replaced sucrose as a sugar source, to better match the carbohydrate source *in alvo*, and the protein supply was changed to pollen pellets in order to match the natural diet of the bumblebees.

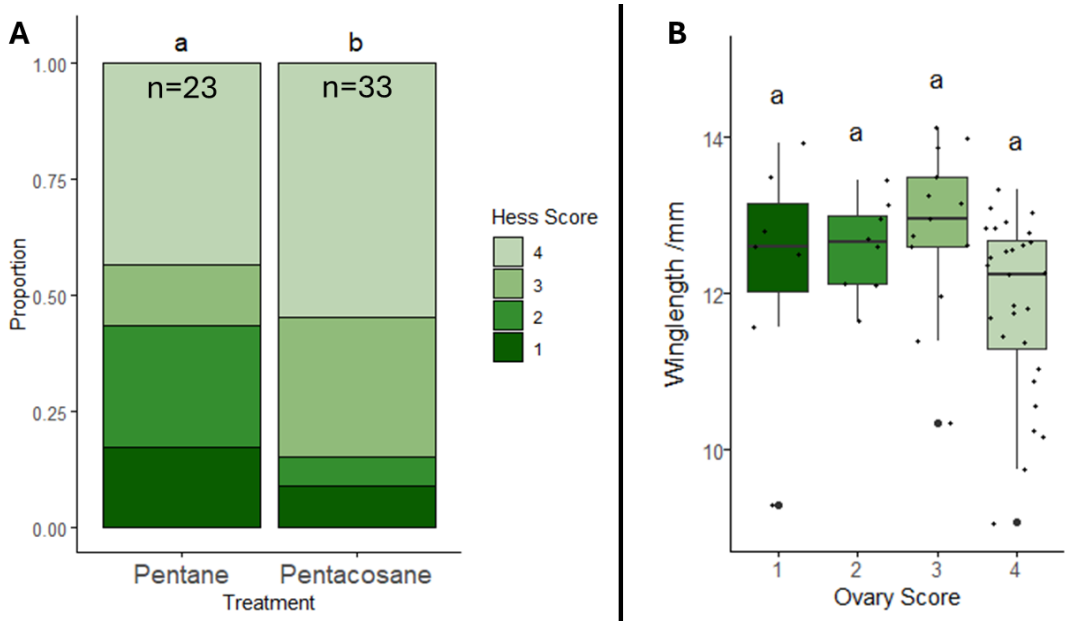


Figure 4.17 – Showing the effect of nC25 and forewing length on ovary activity in *B. terrestris* workers. **A**: the effect of treatment on the ovary activation of *B. terrestris* workers exposed to queen pheromone pentacosane or solvent control. Ovary activation is measured via modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid). The y-axis shows proportion of bees at a given Hess score, and the x-axis shows treatment of pentacosane or pentane solvent-control. Significance letters given as P<0.05, calculated via CLMM and post-hoc Tukey pairwise test. **B**: the effect of ovary score on forewing length in the same dataset. The y-axis shows forewing length in mm, and the x-axis shows ovary score as calculated via modified Hess score. Significance is shown as letters (P<0.05). Although there is a significant difference between treatments in panel **A**, the model suggest winglength to be a better predictor of ovary activity, which does not produce any significant interaction.

4.3.2.2 Pentacosane-mediated reproductive constraint is not reproducible in an *in cavea* setup in *B. terrestris* in the same manner as QMP in *A. mellifera*

At a much later point in methodological development, after the switch to pollen as a protein source, and BioGluc as a sugar source, the response of *B. terrestris* workers to nC25 was retested, including QMP as a secondary investigation.

As can be seen in Figure 4.18**A**, the changes in methodology were not enough to bring about repression of ovaries either via 1Qe nC25 or 0.25Qe QMP per day (χ^2=0.5930, df = 2, P=0.743; pairwise test comparisons in Table 46 in appendices).

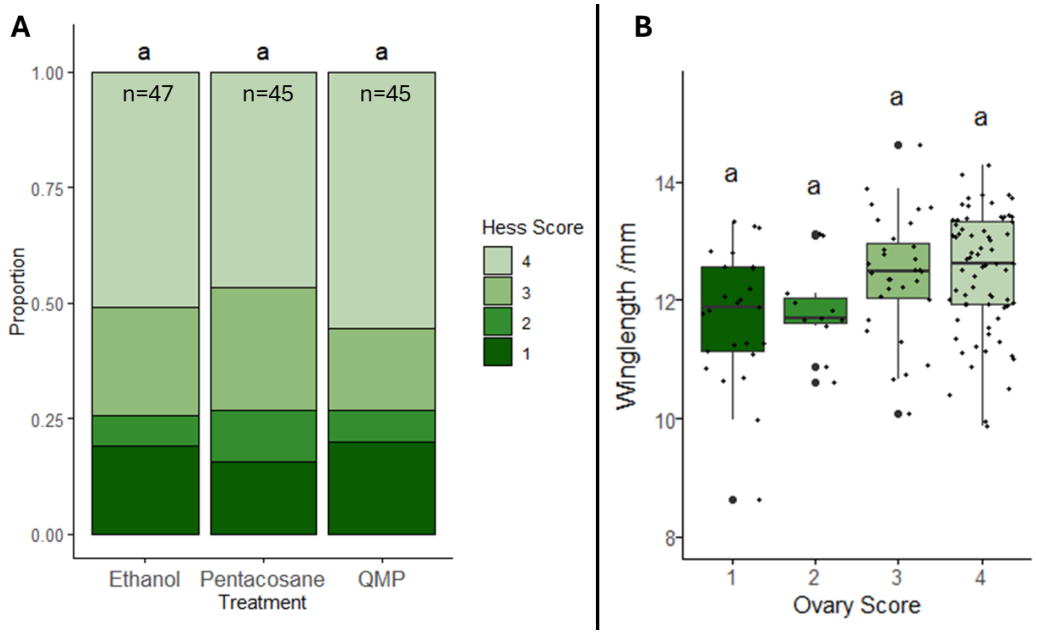


Figure 4.18 – Showing the effect of nC25, QMP, and forewing length on ovary activity in *B. terrestris* workers. **A**: the effect of 1Qe native queen pheromone pentacosane per day and honeybee 0.25 Qe QMP on ovary activity per day, determined by modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid) in worker *B. terrestris*, using a more biologically realistic setup. The y-axis shows proportion of given ovaries at each Hess score, and the x-axis shows the treatment: ethanol and pentane control; pentacosane *B. terrestris* queen pheromone; and QMP. Significance given as letters (P<0.05) calculated by CLMM and post-hoc Tukey tests. **B**: the interaction between forewing length and ovary score in the same dataset. With the y-axis showing forewing length in mm and the x-axis showing ovary score determined by modified Hess score. Significance given as letters (P<0.05) calculated via GLMM and post-hoc Tukey tests. In both cases there is no significant interaction of treatment and ovary score, or winglength and ovary score.

In this model, forewing length (as a proxy for bumblebee size) showed as a greater predictor for the dependent variable, and this is shown in Figure 4.18**B** (χ^2=5.8415, df = 1, P=0.016), though notably with the opposite trend as seen before; however in post-hoc pairwise tests, ovary score and forewing length did not statistically significantly correlate (pairwise test results shown in Table 47).

There was however a significant difference in mortality between the bees in cages exposed to the different pheromones (as shown in Figure 4.19). In this figure we see that QMP has had a negative impact on survival (similar to that demonstrated in honeybees in chapter 3) and that pentacosane has had a positive impact on survival such that they are significantly different from one another (χ^2=14.88, df = 2, P<0.001; results of individual pairwise tests in Table 48, in the appendices).

The results of this experiment imply that the reproducibility of pentacosane-mediated reproductive constraint in *B. terrestris* is not great enough *in cavea* to be able to use this model as a method for investigating the mechanism of action of this pheromone in this organism.

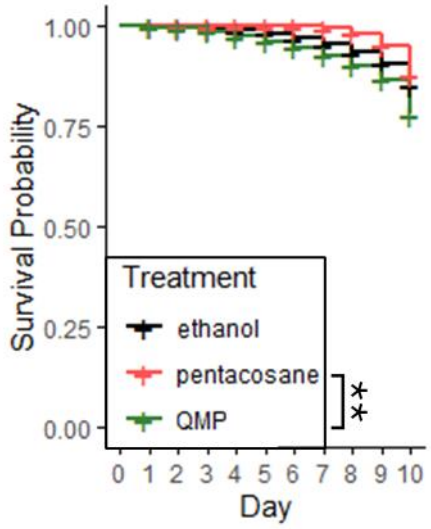


Figure 4.19 – Showing a Kaplan-Meier survival curve for the data presented in Figure 4.18. The y-axis shows survival probability and the x-axis day of experiment. The colours represent the different treatments. There is significant difference in mortality between the two pheromone treatments.

4.3.2.3 *B. terrestris* response to QMP

QMP has been shown to induce ovary repression in *B. terrestris* workers (209). In order to investigate the mechanism of action of this activity, and compare it to the mechanism of action

in *A. mellifera*, it must first be established that we can reproduce this repressive effect in an *in cavea* setup in our lab.

4.3.2.3.1 QMP-mediated repression of *B. terrestris* workers is not reproducible

B. terrestris workers were either exposed to 1Qe QMP/day or ethanol solvent control for 7 days. This relatively high concentration of QMP was chosen in order to maximise the chances of a strong phenotype, and is consistent with the experiments published by Princen et al. (128).

However, as can be seen in Figure 4.20**A**, QMP was not able to induce a repressed ovary phenotype in worker bumblebees (χ^2=3.0869, df = 1, P=0.079).

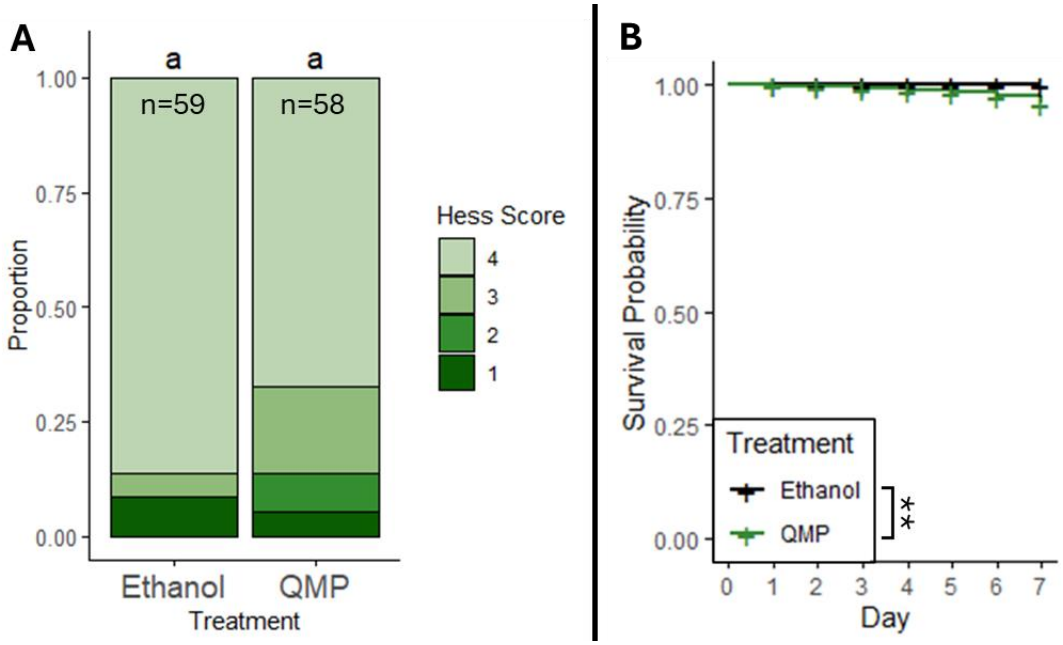


Figure 4.20 – Showing the effect of QMP on ovary activity and mortality in *B. terrestris* workers. **A**: the effect of QMP on ovary activation, as measured by modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid), in *B. terrestris* workers. The y-axis shows proportion of bees at a given Hess score, and the x-axis shows treatment of QMP or ethanol solvent-control. Significance given as letters (P<0.05) calculated via CLMM and post-hoc Tukey pairwise tests. **B** shows the Kaplan-Meier survival curve of the same data with the y-axis showing survival probability, and the x-axis showing day of experiment. Treatments represented by colour. In both **A** there is no difference between the two treatment groups, but in **B** there is a significant difference in mortality between treatments.

As can be seen in Figure 4.20**B**, this did produce a significant effect on survival probability however, with QMP producing a statistically significant increase in mortality relative to ethanol control (χ^2=9.7041, df=1 , P=0.002), consistent with that effect seen in honeybees in chapter 3. Implying that the marginal, statistically non-significant difference seen in panel **A** may be as a result of sub-lethal mortality effects on the surviving bees, which we would expect to see if there is a significant mortality effect of treatment.

This data implies that the repressive activity of QMP on *B. terrestris* workers is not as easily reproducible as the QMP-mediated reproductive repression brought about by this pheromone in *Apis mellifera*.

4.3.2.3.2 Colony-of-origin affects the susceptibility of bees to ovary activation

The data laid out in aggregate in sections 4.3.1.5 and 4.3.1.6, was from the same experiment in which the number of bees in a cage were exposed to different pheromone treatments as well, which has been redrawn in Figure 4.21, Figure 4.22, Figure 4.23, and Figure 4.24 (N.B. these are all of the same dataset).

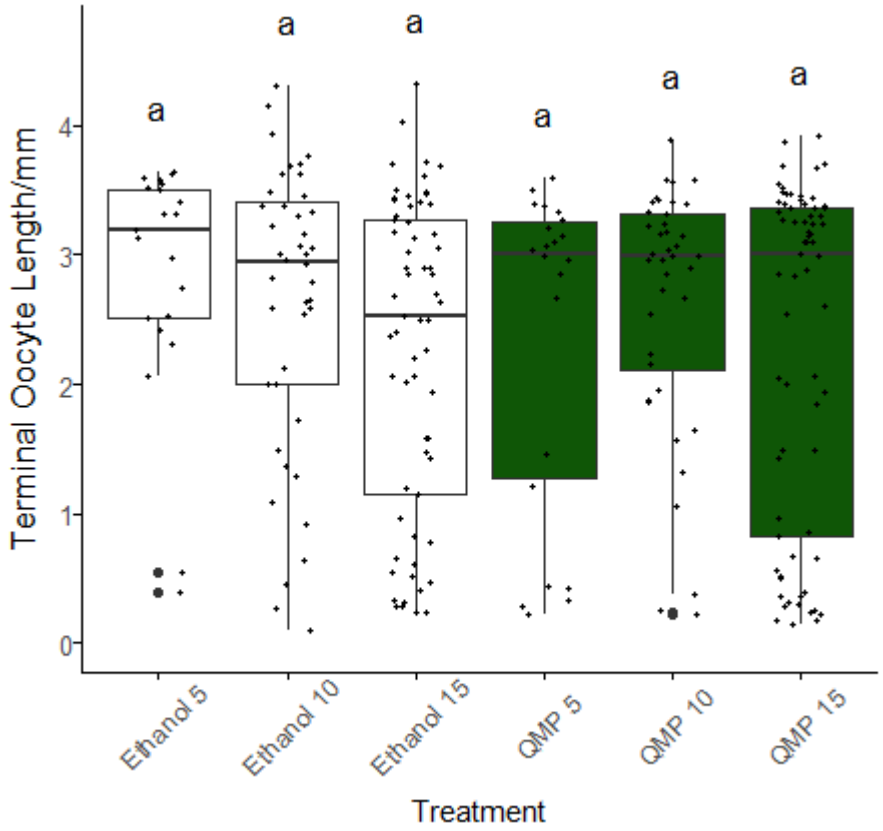


Figure 4.21 – Showing the effect of number of bees in a cage on ovary activation via measuring of the largest terminal oocyte in an ovariole of a given bee's ovary in *B. terrestris* workers. The y-axis shows the length of the terminal oocyte in mm, and the x-axis shows treatment group: Ethanol/0.25QeQMP with regards to if the bees had been exposed to honeybee QMP or solvent control; and 5/10/15 for the number of bees in each cage. Significance is given as letters ($P<0.05$) calculated by GLMM and post-hoc Tukey pairwise tests. The graph shows no effect of bees per cage on ovary activation.

The effect of treatment with QMP in these cages was included in order to investigate whether the social effects of different numbers of bees per cage produced attenuation to QMP's ability to induce repression of worker ovaries. This is based on research which has shown that hierarchies of dominant submissive workers form in the absence of a queen (285), and that the presence of a queen is necessary for worker ovary repression(289).

Figure 4.24 shows the data of Figure 4.21, using an alternative statistical approach of linear regression.

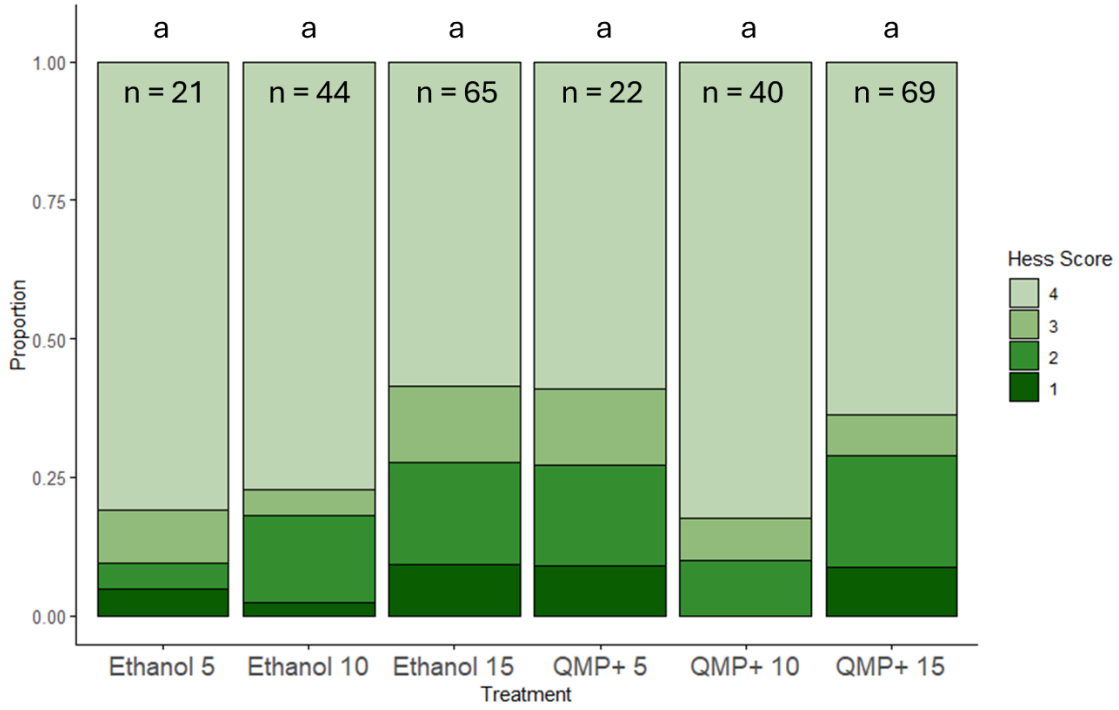


Figure 4.22 – Showing the effect of number of bees in cage on ovary activation via measuring of ovary development measured by modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid) in *B. terrestris* workers. The y-axis shows proportion of each treatment group categorised by modified Hess score. The x-axis shows treatment group: Ethanol/0.25QeQMP with regards to if the bees had been exposed to honeybee QMP or solvent control; and 5/10/15 for the number of bees in each cage. Significance given as letters ($P<0.05$) and calculated by CLMM and post-hoc Tukey pairwise tests. The graph shows no effect of bees-per-cage on ovary activation.

In both Figure 4.21 and Figure 4.22 we see the lack of any effect of treatment on ovary activity, separated out by numbers of bees in each cage (as previously demonstrated in aggregate in section 4.3.1.6) (Figure 4.21: $F=2.1151$, $df=5$, $P=0.062$; results of individual pairwise tests in Table 38 in appendices). Although we see in the data shown in Figure 4.22 that the model shows that treatment and number of bees per cage combined is an appropriate predictor of effect ($\chi^2=16.248$, $df=5$, $P=0.00617$; results of pairwise test results in Table 39 in appendices)

Interestingly however, for the data contained in Figure 4.21, the model shows that colony-of-origin of bees in the cages is an adequate predictor of terminal oocyte length ($F=20.317$, $df=3$, $P<0.001$).

This interaction between covariate and dependent variable is shown in Figure 4.23, where there is a significant effect of colony-of-origin on terminal oocyte length between colony D and the other colonies-of-origin (individual pairwise test results laid out in Table 40).

When separated out by treatment in Figure 4.24, we see that the ethanol control exhibits a statistically significant slight negative correlation between ovary activity (as measured by terminal oocyte length) and numbers of bees per cage (R^2=0.04; F = 5.66, P=0.02).

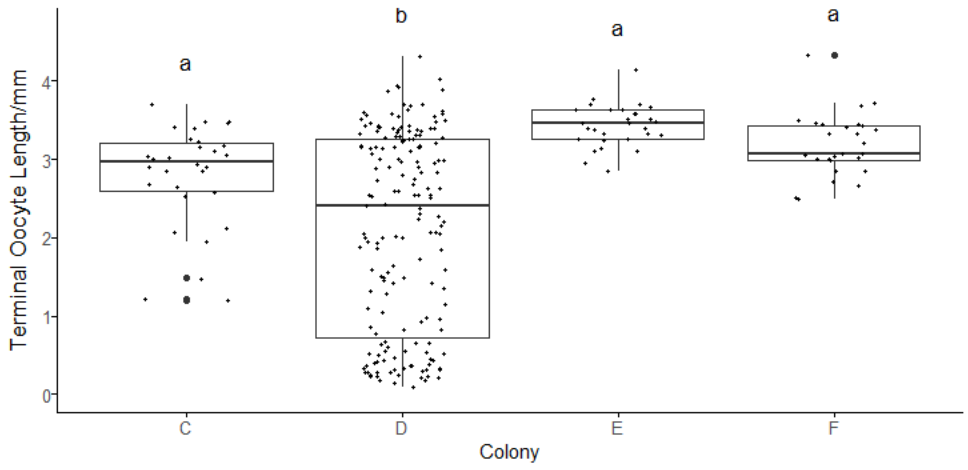


Figure 4.23 – Showing the effect of colony-of-origin on the ovary activation of the bees used in the experiments investigating number of bees per cage in *B. terrestris* workers. Ovary development is measured as the length of the largest terminal oocyte in a given ovary of a given bee. The y-axis shows the length of the terminal oocyte in mm while the x-axis shows the colony-of-origin of the bee, as identified on receipt of colony by identification with letters. Significance given as letters (P<0.05) calculated via GLMM and post-hoc Tukey pairwise test. The graph shows that colony-of-origin can be a significant predictor of ovary activation.

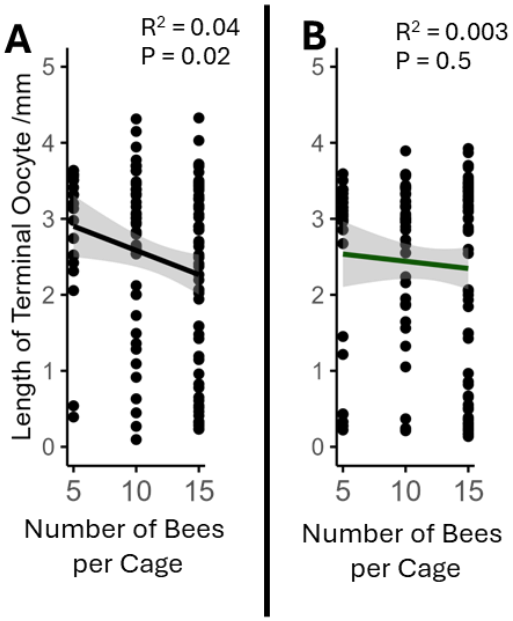


Figure 4.24 – Showing scatter graphs and linear regressions for the data shown in Figure 4.21, showing the response of *B. terrestris* worker ovary activity to treatment and numbers of bees per cage. The y-axis shows Length of terminal oocyte and the x-axis shows number of bees per cage. **A** shows bees exposed to ethanol solvent control. **B** shows bees exposed to QMP treatment at 0.25Qe/day. Ethanol exposed bees show a significant small negative correlation between ovary activity and number of bees per cage, while QMP bees show no significant correlation.

4.3.2.3.3 Difficulty of reproducing QMP-mediated ovary repression in *B. terrestris* not result of dosage

Given that other organisms show that QMP acts in a dose-dependent response with regards to ovary repression (148). The concentration of QMP used to investigate the effect of QMP-mediated reproductive repression on bumblebees is an important variable to control.

Especially as in honeybees, workers appear to respond to QMP in a U-shaped response curve by an unknown mechanism (Unpublished, (104)), simply using very high concentrations of QMP would not necessarily guarantee a more marked phenotype.

As a result, a QMP dose-response was carried out in *B. terrestris* workers. This was carried out twice, at different points in the methodology development.

The first attempt is shown in Figure 4.25**A**, where there was no impact of treatment on ovary repression (χ^2=7.722, df=6 , P=0.259; results of pairwise tests in Table 43 in appendices); though the impact of colony-of-origin was significant (χ^2=4.898, df=1 , P=0.0269).

Once the methodology had been more refined, the experiment was repeated with larger numbers of bees, and with fewer QMP concentrations (in order to maximise the number of bees per treatment), the results of which are shown in Figure 4.25**B**. This figure shows no relationship between treatment and ovary activation (χ^2=4.429, df=3 , P=0.219; individual pairwise test results in Table 44 in appendices).

The result of these experiments shows that the difficulty in reproducing QMP-mediated reproductive constraint in *B. terrestris* workers cannot be explained as a result of QMP dose issues.

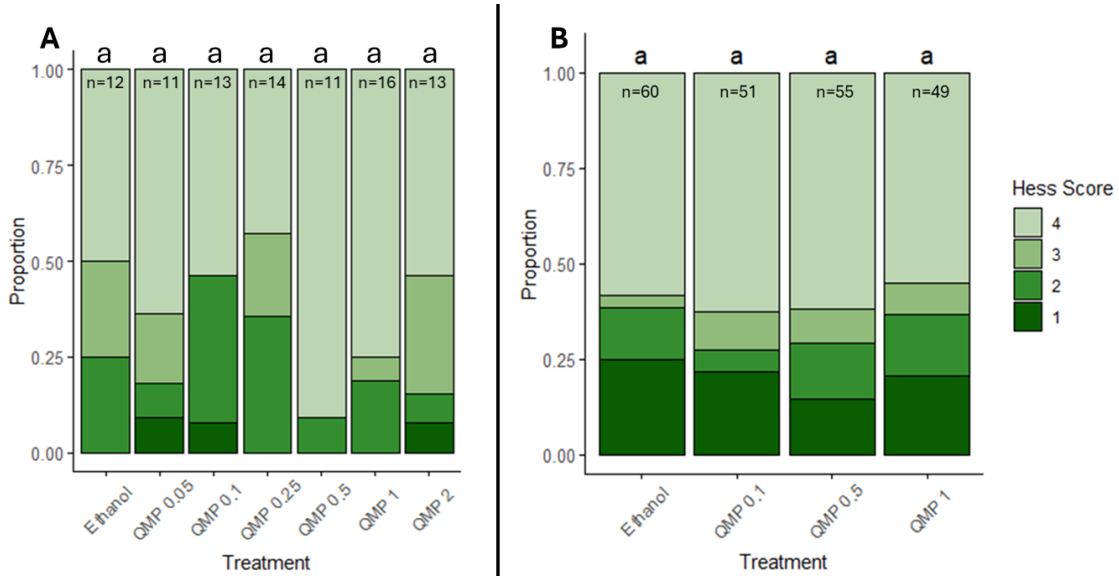


Figure 4.25 – Showing the effect of QMP concentration on ovary activity as measured by a modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid), across two different experimental repeats shown in **A** and **B**. The y-axis shows proportion of bees of a given Hess score, while the x-axis shows the treatment groups: with ethanol being a solvent control and different Qe of QMP given as numbers. Significance is detailed as letters with P<0.05, with independence between the two panels. In both panels QMP failed to produce a significant difference in ovary activity at any concentration.

4.3.2.3.4 Starvation-induced stress does not facilitate QMP-mediated repression in *B. terrestris* bumblebee workers

A possible explanation for the lack of QMP-mediated repression seen in the previous sections may have been that the stress of the bees was too low, and that the QMP-mediated repression of ovaries in this organism previously reported in the literature was a product of the sub-lethal toxic effects of QMP. In this scheme we would therefore expect to see greater QMP-mediated repression in bees which have already been stressed.

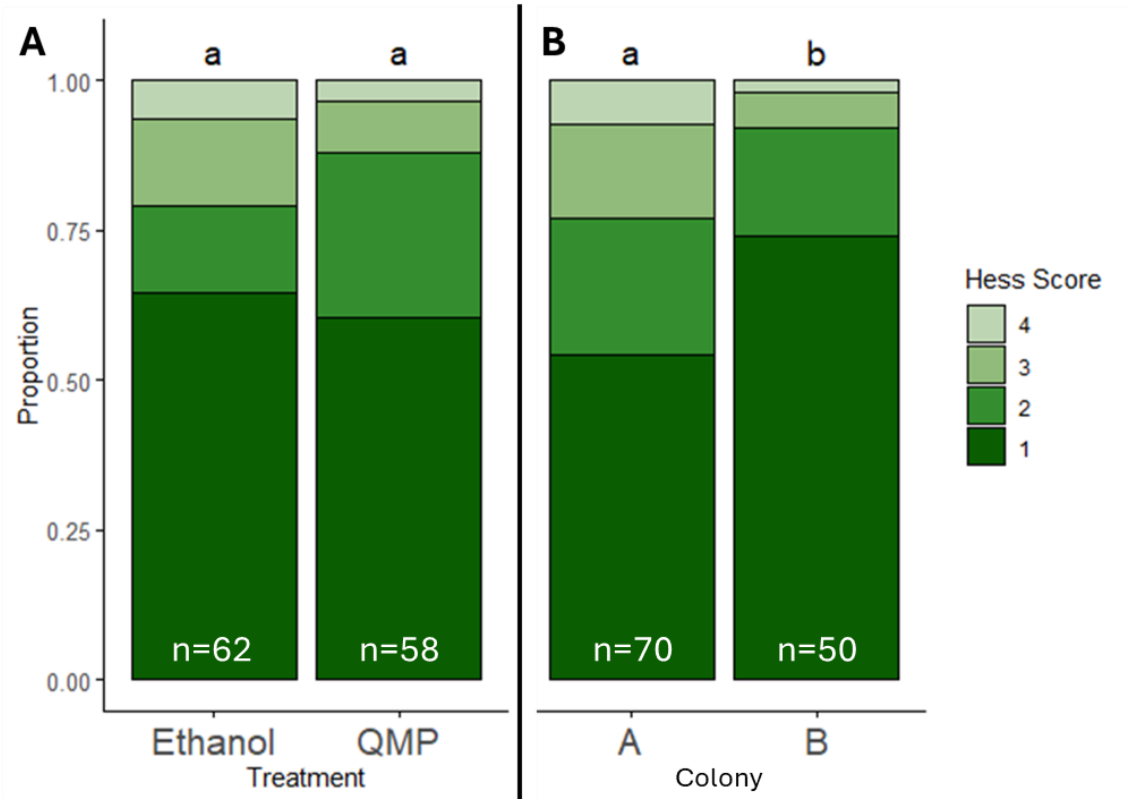


Figure 4.26 – Showing the effect of A: treatment, or B: colony; on the ovary activation of *B. terrestris* workers, as determined by modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid) of *B. terrestris* workers which have been fed food with 50% methylcellulose. The y-axis shows proportion of bees with a given Hess score, and the x-axis shows **A**: the treatment applied, either QMP or ethanol solvent-control; **B** the colony-of-origin of the bees used, given as letters assigned on receipt of colony. Significance is given as letters ($P<0.05$), calculated via CLMM and post-hoc Tukey pairwise test. Treatment had no significant effect on ovary activity, but colony-of-origin did.

In order to test this hypothesis, a less protein-rich pollen mixture was developed (see section 4.3.1.8); and the medium quality mixture was given to bees being exposed to QMP at 0.25Qe QMP/Day, and bees exposed to solvent control.

As can be seen in Figure 4.26**A**, this lower protein mixture did not bring about statistically significant repression of *B. terrestris* worker ovaries (χ^2=0.2087, df=1 , P=0.578). In this model colony-of-origin was shown to be a better predictor of ovary activity, which is shown in Figure 4.26**B** (χ^2=4.326, df=1 , P=0.0376).

In this experiment there was also no effect of treatment on mortality, although mortality in both treatments was higher than usual, probably as a result of the lower food quality. This is shown in Figure 4.27 (χ^2=0.0014, df=1 , P=0.97).

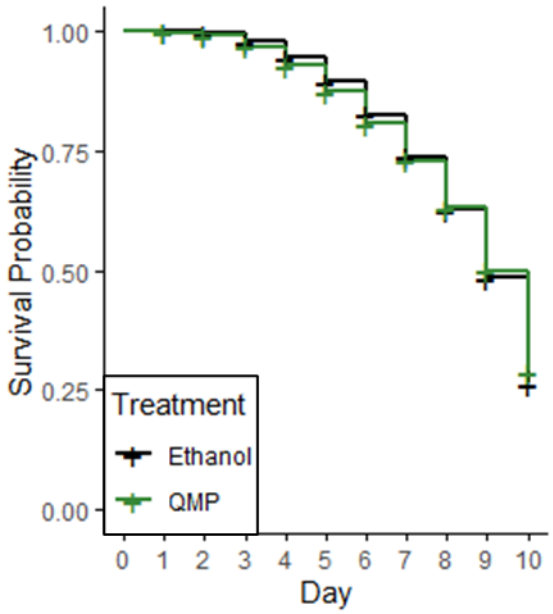


Figure 4.27 – Showing a Kaplan-Meier survival curve of the bees used in Figure 4.26. The y-axis shows survival probability and the x-axis day of experiment. Treatment is shown as colour. There is no effect on survival as a result of treatment.

4.3.2.3.5 Age Matched Bees

A possible explanation for the lack of QMP-mediated constraint in *B. terrestris* workers demonstrated in previous sections, could be that the workers in each of the previous sections were not age-matched. I.e. they were of various ages from different parts of the colony lifecycle, and this may result in different sensitivities to QMP (226).

This may also explain the colony-of-origin effects seen in previous sections, as the colonies were of different ages when worker bees were sampled, and so presumably from different stages of the lifecycle.

In order to investigate this, workers were age matched at day 1 post-eclosure as adults before being placed in cages for the experiments. These cages were then either exposed to QMP at 0.1QeQMP/day, or ethanol solvent-control. This concentration was selected as it reflects a similar per bee concentration of QMP as that used in Princen et al. (128)

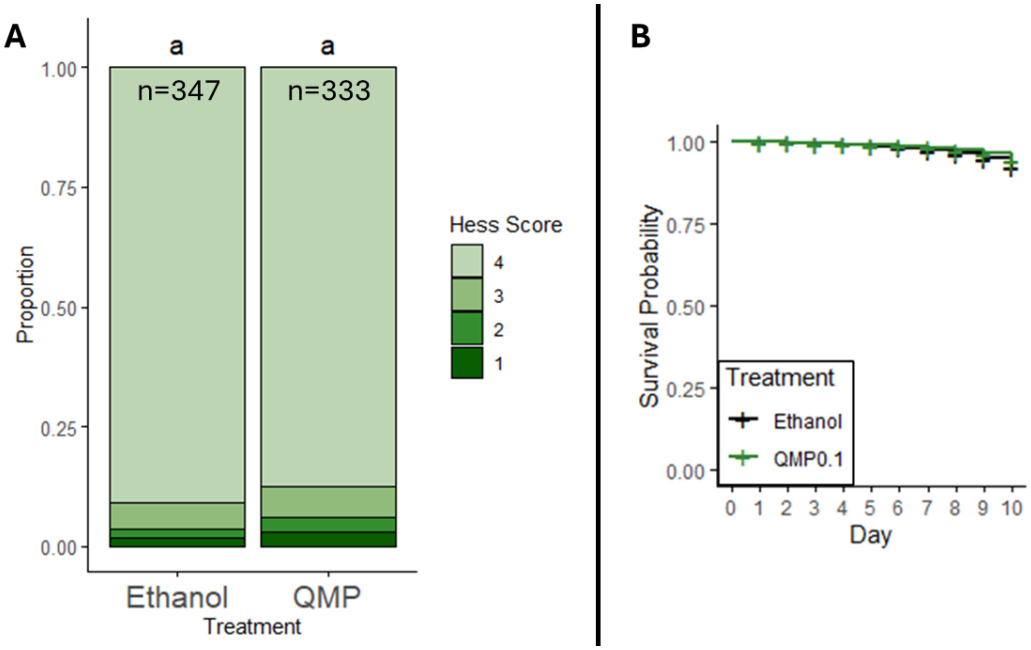


Figure 4.28 – Showing the effect of QMP on ovary activity and mortality of *B. terrestris* workers **A**: the effect of QMP on the ovary activity, as measured by modified Hess score (1 = can distinguish between oocyte and nurse cells but nurse cells larger than oocyte; 2 = oocyte same size as or larger than nurse cells; 3 = nurse cells starting to degenerate; 4 = fully developed ovum ready to be laid), of age-matched *B. terrestris* workers. The y-axis shows proportion of bees with a given Hess score, and the x-axis shows the different treatments: QMP or an ethanol solvent-control. Significance is given as letters ($P<0.05$) calculated via CLMM and post-hoc Tukey pairwise test. **B** shows the Kaplan-Meier survival curve of the same data. On the y-axis is survival probability and the x-axis day of experiment, with treatment as different colours. In both panels there is no significant difference between treatments.

As can be seen in Figure 4.28**A**, there was no effect of QMP on ovary activity in these young age-matched workers ($\chi^2=0.4429$, df = 1, $P=0.506$). There was also no statistically different probability of survival between treatments ($\chi^2=3.4815$, df = 1, $P=0.062$).

The results of this experiment conclusively show that young age-matched bees are not susceptible to QMP-mediated reproductive repression of ovaries in an *in cavea* context.

4.4 Discussion

QMP has been shown to repress reproduction in many species (148, 149, 288), despite the fact that neither it, nor its components, have been identified as a native pheromone of these organisms. Whether the mechanism of action by which QMP is able to repress reproduction in these diverse species is the same or different, informs us of the evolution of this pheromone mixture in the honeybee.

QMP may have evolved to take advantage of ancestral mechanisms for control of reproduction as part of an arms race (169): in which case the mechanism of action of this pheromone mixture would be widely similar in the different organisms in which it acts. It may not have the same mechanisms however, and the first step to elucidating the mechanism of action of this pheromone is to establish a model in which QMP-mediated repression of ovaries can be investigated in greater detail. If the mechanisms are different between species, or between honeybees and other species, it indicates that sensory exploitation is not the manner through which QMP evolved to repress reproduction, and that the sender precursor theory is more likely the path of evolution of these pheromones.

The *B. terrestris* bumblebee provides a logical choice. It has been shown to have been reproductively repressed by the presence of QMP (209). It also has a relatively well characterised genome, making it simple to investigate gene expression and molecular mechanisms within. They are relatively closely related to *A. mellifera*, and share a single origin of eusociality (7). They are also easily obtainable.

In order to use this organism as a model to investigate the mechanism of action of QMP, the insects must first be established in our lab setup. The *in cavea* setup used in honeybee assays provides a simple and easily manipulatable environment in which to investigate the effect of pheromones on insects, and so attempts to replicate this setup in *B. terrestris* workers was made.

In alvo experiments contain many potentially different and uncontrollable confounding variables, which reduce the strength of any conclusion drawn; whereas *in cavea* experiments allow us to investigate very specific elements of the biology of the organism, without the interpretive issues that arise.

The methodological development pathway, shown through section 4.3.1, allowed for the investigation of pheromone activity shown through section 4.3.2. However, much of these experiments were carried out alongside one another, and so interpretation of these data must be taken within the context of the methodological development undertaken.

4.4.1 The lack of QMP and nC25 mediated activity in the *in cavea* setup

Throughout the experiments, only once was one of the two queen pheromones, QMP and nC25, able to induce changes in ovary activation, and this was in contrast to that published in the literature (Figure 4.17; in this instance, it is possible that the ovary effect measured was as a result of unmeasured colony-of-origin factors).

4.4.1.1 The biological relevance of previously published findings

The lack of ease with which reproducibility can be demonstrated of the QMP and nC25-mediated repression of ovaries in *B. terrestris* previously published, raises questions about the biological relevance of this reported response.

The QMP-mediated reproductive constraint in *A. mellifera* workers is able to occur in conditions which are very different from natural biological conditions (i.e. *in cavea* (47, 104, 132)). The *in cavea* experiments undertaken to show this also isolate the bees from many other social effects, such as worker policing. These data show, therefore, that this pheromone is a core mechanism for the maintenance of reproductive constraint in the honeybee.

That neither the proposed native queen pheromone nC25, or QMP, are able to bring about a clear repression of ovaries in *B. terrestris* under any circumstances tested in this thesis implies that the effect of these pheromones for reproductive constraint *in alvo*, are relatively unimportant. I.e. that the effect size of these pheromones on reproductive constraint is small enough, or insignificant enough relative to other mechanisms (such as the physical dominance hierarchies shown to be present in *B. impatiens* (283)), that they are biologically irrelevant. (or, in the inverse, that if these pheromones were biologically relevant, that they would be clearly and easily reproducible in this organism even under sub-optimal conditions)

The 2018 meta-analysis by Holman (290) highlights both the effect size disparity of various pheromones across different species, including the small effect size of two papers showing the response of *B. terrestris* bumblebees to nC25: Van Oystaeyen 2014 (46), and Holman 2014 (88), although the methodology of this meta-analysis has been disputed (291). These two papers used markedly different methods to investigate nC25-mediated reproductive constraint: Van Oystaeyen applied high concentrations of nC25 to small young colonies of ~20 workers, and measured the ovary activity via a modified Hess score. Whereas the Holman paper investigated large colonies of ~300 bees reportedly past the competition point (this observation was made based on bee number, not the behaviours associated with competition point), with very low concentrations of nC25, and measured the ovary activity via oocyte count per ovary. This paper also identified bees of different sizes by general observation, not by measurement, and then

uses those observations to inform conclusion and as a variable in statistical modelling; which is not a particularly robust methodology, especially considering the ease with which measurements could have been taken.

A third, more recent, paper showed the efficacy of QMP as a mechanism of ovary repression in *B. terrestris* workers, but failed to reproduce the findings that nC25 acted in a way which repressed the ovary activity of workers directly (by measurement of modified Hess score), however they did demonstrate the loss of fertility associated with reproductive constraint through the proximate method of "eggs laid" (209). This possibly indicates that different methods of investigating reproductive constraint for different pheromones should be utilised, e.g. instead of utilising modified Hess scores, more direct measurements such as eggs laid by workers should be used. It also possibly suggests the mechanism for repression at a physiological level is different for these two pheromones nC25, and QMP in *B. terrestris*.

In this third paper, the methodology similarly used microcolonies of 20 bees per colony, and included comb and brood of all stages.

In all of these papers, the bees were kept in some manner in their colony-of-origin, and presumably brood was still present in each colony for the former two papers, it was deliberately included in the third. Other potential queen pheromones or substances may also have been present during the course of the experiments (though this was not reported in any paper). No paper used fewer than 20 bees per microcolony. All of these factors combined obfuscates any possible synergistic effect of extraneous forms of reproductive constraint, and it is possible that nC25 is only able to bring about repression as a contributing factor to other forms of reproductive constraint. The utilisation of *in cavea* methods, which are more sterile, would perhaps give a greater weight to any conclusions drawn about the nature of nC25 as a queen pheromone, however the absence of reproducibility demonstrated in this chapter for these pheromones using a highly sterile *in cavea* setup strongly implies that these reported repressive effects are not reproducible using only the reported queen pheromone.

Also, the use of oocyte regression as a proxy for ovary repression, as described in the Van Oystaeyen paper (46) is unusual. It has been noted that oocyte regression in bumblebees is only able to occur in active ovaries (282). This is in line with observations made during the course of the experiments of this chapter, where oocyte regression only occurred in the most active ovaries.

This is because oocyte regression is the process by which fully developed eggs are resorbed in the ovary, rather than laid (131). In a normal Hess score system, only the smaller scores are named as inactive (usually Hess scores of 0-2 as defined in this Thesis), whereas oocyte

regression is invariably named as an addendum to higher ovary scores (e.g. the score system used by Duchateau et al. of I, II, III, III^{+}, IV, for increasing ovary development with IV^{R}, being regressed oocytes (279)). The fact that this is being used as the metric to measure ovary repression is arguably inappropriate, and indeed it is not one of the five types of reproductive constraint identified by Khila et al. (292), and the method used by Holman of measuring the number of eggs per ovary (88) may be a more representative measure of ovary activity, although this has its own problems as mentioned in section 4.1.1.3.

Regardless of the issues with these papers, the fact that the results from the *in alvo* experiments undertaken in section 4.3.2 were not able to be replicated *in cavea* implies that nC25 is not as core a mechanism as suggested in these former two papers. This is perhaps because it combines with other signals present in the hive to function; or that there is some unreported social effect of bumblebee workers on one another which allows for the induction of reproductive constraint via nC25 exposure (social effects are known to cause reproductive constraint in bumblebee workers, as physical presence of queen is known to repress active worker ovaries in pre-competition point hives (289), and in *B. impatiens* this effect requires the physical interaction between queen and worker (281) however this effect has not been isolated from the effect of queen pheromone). It is also possible that the two published examples of nC25-mediated repression of ovary activity are disputable for methodological reasons.

Until a more controlled *in cavea* setup is able to replicate nC25 or QMP activity in *B. terrestris* workers, it is reasonable to call into question its biological relevance.

4.4.1.1.1 Cage Material

Another of the key differences between the work carried out in these papers and that of this thesis, is that the cages used for *in cavea* experiments in our lab setup are autoclavable metal (to allow for reusing of cages between experiments). This is compared to the plastic boxes supplied by the agricultural companies (as in Holman et al. (88), or Van Oystaeyen et al. (46) or custom wooden cages used by and Princen et al. (209)). It is possible that the lack of mobility this produces (e.g. by the inability of the bumblebee workers to climb the walls), or abiotic factors like temperature and humidity maintenance, played a significant part in the behaviour and biology of the workers, interfering with pheromone activity.

In order to narrow down the possible negative impact that the presence of comb and brood, and the material of cage effect on the effective establishment of *in cavea B. terrestris* experiments, more methodological development must occur.

4.4.1.2 Diversity of queen pheromone

Since the 1960s (293), where honeybee QMP was first indicated via extraction of mandibular glands, more and more components of honeybee QMP have continuously been discovered. Even with the discovery of the five principal components (108), continued investigation into QMP's complex mixture are still being undertaken, with papers still being published (47).

Although the complexity of QMP is remarkable compared to other queen pheromones in other organisms, the idea that other hymenopteran queen pheromones are single molecules is also questioned, with the same Van Oystaeyen paper as earlier (46) describing several compounds which work across species and within the same species.

The suggestion therefore that the singular CHC of pentacosane is the only queen pheromone, is crucial to avoid. Very few substances have been shown to have been tested in *B. terrestris*, and it is reasonable to suggest that queen pheromone in this species could be a complex mixture.

The use of gas chromatography – mass spectrometry (GC-MS) has frequently been used to isolate and investigate potential pheromones, whether by the extraction of glands and processing of the compounds contained therein, or by taking washes of the exoskeletons to extract CHC mixes (47). Understanding which of the compounds extracted is responsible for reproductive constraint if any, and in which any potential combination is necessary, is a large, but biologically important undertaking.

4.4.1.3 The role of colony-of-origin

One of the most significant covariates throughout the results of this chapter, was the impact of colony-of-origin as a greater predictor of ovary activation than treatment. Typically this manifested as greater variation in ovary activity, particularly towards the lower end (i.e. that there was much more presence of inactive ovaries in certain colonies).

The experiments carried out throughout this chapter, were often carried out at different times of year. Primarily, however, they were carried out through the winter months, as the summer was reserved for honeybee work, and the commercial availability of bumblebee colonies for use in all-year greenhouses as pollinators (208); makes it possible to obtain these colonies even when wild colonies are not available. The ages, and stages of development for the colonies used however, was dependent on the availability of the supply from Agralan.

As a result the colony effects seen in this chapter could be as a result of the genetics of the colony, or as a result of age/development period from when the colony was obtained.

4.4.1.3.1 Colony Genetics

Best shown in Figure 4.23, the significant impact of colony-of-origin might simply be a result of the genotype of a colony determining its sensitivity to queen pheromones. This is seen in honeybees, where sensitivity to QMP can be manipulated via selective breeding (108). It might also be that different colonies also have varying amounts of latent ovary activation amongst workers (which is also observed in honeybees between subspecies, such as the greater degree of ovary activation amongst the cape honeybee *A, mellifera capensis* vs other subspecies (171))

Given that the colonies obtained for the experiments are commercially produced and reared artificially, we would expect to see less variation than in wild bumblebee colonies (as with all domestically reared animals (294).

The well-characterised nature of the bumblebee genome (295), and the ease with which genetic characterisations can be made, make this an ideal mode of investigation for the potential genetic variation between colonies. By taking samples of the bees used while dissecting, a library of tissue of bees from different colonies could be gathered to investigate the effect of colony genetics, as well as being able to correlate this with ovary activity, sensitivity to pheromone, forewing length (as were measured in this thesis); or any other biotic factor for ease of analysis.

4.4.1.3.2 Possible effect of colony age on pheromone sensitivity

When the colonies were procured, there could be anywhere from between five workers present, to 100 or more. Often, when several colonies were ordered at the same time, they were of greatly different sizes. Unfortunately, due to the proprietary nature of the method used to artificially rear the colonies by Agralan, we were not able to ascertain the precise method used, and so were never sure of the age, or if that age and size were always correlated in the same manner between different colonies (though it is probably reasonable to use colony size as a proxy for age in this species).

Given the well-defined three-stage colony cycle that *B. terrestris* go through, in which there is a markedly different reaction of the workers to the queen, and an overall breakdown of reproductive constraint progressively throughout the colony lifecycle, as a result one would expect that bees become less sensitive to queen pheromone as a reproductively repressive chemical as they age. The age of the colony seems a very important factor to be able to control.

In order to account for the effect of colony age, the number of bees in a given colony at time of arrival might be a way to gather a baseline, but this assumes that the manner of rearing the

colonies by Agralan matches the biological reality of a wild colony. Direct communication with Agralan to have them report the age of each colony might be a more foolproof method of colony aging, and so this can be investigated as an important effect on ovary activity and pheromone sensitivity.

It is known that the bumblebee queen engages in many direct behaviours which inhibit worker egg-laying (285). She polices bees individually, punishing them as they lay and by eating the eggs of worker-laid eggs (279). It has also been shown that the physical presence of the queen is necessary for reproductive constraint of workers in the early lifecycle of a colony, and that pheromone alone is insufficient (281). When the colony is small, and she has stopped foraging, with relatively few bees to manage, she is able to be a prominent physical presence for each individual worker. However, as the colony life-cycle progress, and considerably larger numbers of bees are present, many of whom are spending large amounts of time outside the hive foraging, her individual contact with bees decreases, and her control over those bees decreases.

As the colony ages, the action of queen pheromones as a method for reproductive constraint would become far more important. We also see the switch to competition between workers and between the workers and the queen increase over this period (131, 279). It might be that queen pheromone sensitivity remains the same over this period, or that it starts low (as it is unnecessary due to the presence of the queen) and increases in sensitivity over time (so as to maintain reproductive constraint for longer, to maximise the reproductive fitness of the colony). Or it could be the opposite, that queen pheromone sensitivity is higher at the start (due the importance of cooperation for increasing the size of the colony, in order to maximise reproductive fitness), and that it becomes weaker with time (as selfish behaviour of the workers becomes more beneficial to their own reproductive fitness). This could be achieved by exposing workers of colonies at specific ages to pheromone and determining if this is able to bring about repression. Or alternatively, using queenless microcolonies of different ages and using queen pheromone to induce repression, then measuring the effect size of this between different aged colonies.

Given that it is known that reproductive constraint of workers reduces with time in *B. terrestris* colonies, the sensitivity to the queen pheromones shown to act in this species would give significant insight into physiological and metabolic changes that occur, and bring about, the lifecycle changes of this organism.

4.4.1.4 Age effects of the bee itself

Throughout the development of the *in cavea* assays in this chapter, age-matching of bees, particularly the use of callow bees for assays, was trialled several times, with a broad colony-wide approach used for the final experiment shown in Figure 4.28**A**. However, this method used relatively young colonies, for which all of the workers of a given colony were chilled to anaesthetise and mark, and then subsequently any unmarked bees were removed every 24h and used for experiments. It was proactively decided to avoid chilling bees used for assays, as this could significantly affect behaviour/ovary activity. This was able to standardise the age of the individual bee, but not the colony as a whole. It also prevented the bees from spending very much time in their colony before being used for *in cavea* experiments, and guarantees that only callows are used for the experiments.

Evidence of the changes in sensitivity to queen pheromones with age have been observed in *A. mellifera*. In this species it is known that older workers are no longer attracted to QMP (226). It has also been shown in Chapter 3 that they do not need to be exposed to QMP for the first few days of life in order for reproductive constraint to be maintained. Given that the *B. terrestris* workers exposed to QMP in Figure 4.28**A** did not exhibit any degree of sensitivity to this pheromone, but that the workers in Princen et al. (209) were older and did exhibit sensitivity to QMP, it might be a reasonable observation that age of individual bee is also a factor in pheromone sensitivity. If this is true in this way, then this would be evidence that the response to QMP in *B. terrestris* follows an inverse pattern of sensitivity to QMP than in its native *A. mellifera*.

The age-matching setup used in this chapter could be adapted to age match bees in a way that allows for bees of a given age to be used for experiments, rather than callows in order to investigate this approach in detail.

4.4.1.5 Social effects

It was noted in Figure 4.21 that, while not statistically significant, there was a trend towards larger numbers of inactive ovaries at higher numbers of bees per cage. This effect was used as a justification for using 10 bees per cage, to prevent this phenotype from being a confounding variable.

It is possible however, that the larger numbers of bees per cage are necessary for reproductive constraint in *B. terrestris* bumblebees. The trend seen of decreased ovary activity in some of the bees in these cages may be an example of the natural repression brought about by the beginning of hierarchies (285). No competitive behaviour was observed in these cages

however, though no attempt was made to observe this behaviour, and no behavioural observations of any kind were made during the course of experiments. In the future, behavioural observation would be included formally in order to investigate this.

It is known that worker hierarchies form in queenless contexts (279), but it is possible that these hierarchies do not form with fewer workers present. All papers published showing the effect of queen pheromones on *B. terrestris* did so with at least 20 bees (46, 88, 209).

In social situations with few bees, it is more in the interest of the worker to engage in their own reproduction, as the indirect fitness that would otherwise be exhibited by the queen does not apply, and the other workers have equal reproductive potential to the individual. In this scheme more active ovaries and less sensitivity to queen pheromones, would allow them to maximise their own reproductive fitness. When larger, more dominant workers are establishing a queen-like hierarchy however, then these bees would be constrained into submission.

It is also known that workers become more queen-like with their secretions the more active their ovaries (regardless of the presence of queen)(296), which has been suggested as an honest signal of fertility. In these situations, introducing larger doses of queen pheromone than tested in this chapter, might trick workers into sensing the dominant workers to have more reproductive fitness than they do, and so help to maintain ovary repression according to this logic.

In the future, assays investigating the effect of larger numbers of bees per cage versus fewer may be useful to investigate more thoroughly whether the pheromonal mediation of reproductive constraint in this organism is significantly affected by this factor.

4.4.2 Combination of Stressors and Synergy of Signals

It is reasonable to suggest that the lack of research regarding the interaction of various different elements of reproductive constraint in bumblebees may be the root of the problem of reproducibility of nC25 as a queen pheromone. For example it is known in *B. impatiens* that brood produce pheromones, but that these alone are not enough to induce ovary repression in workers (151). This is consistent with the blend of multiple signals needed for reproductive constraint (103), and the complexity of the colony lifecycle in this organism (40, 131, 279).

As has been mentioned previously, it has been suggested that reproductive constraint is a variation of reproductive senescence/diapause, and that there are potentially multiple abiotic and biotic factors which go into the management of a reproductively constrained phenotype (103).

In *A. mellifera* and *B. terrestris*, the role of diapause is well-characterised. In temperate climates, *A. mellifera* reproductive diapause of queens occurs most markedly during the winter, where reproduction completely stops for six months (76).

In *B. terrestris* founding queens enter a reproductive diapause over the winter, before founding in the spring (40). This period is not essential and *B. terrestris* populations have been known to found in the autumn and continue through winter where forage is available (as in many suburban areas) (278).

The proposal that queen pheromones act to coopt this diapause for the purpose of reproductive constraint has not been investigated in *B. terrestris*. However, it may be that the *B. terrestris* workers *in cavea* do not experience the same degree of stress as *in alvo*, particularly with competition with the queen, natural social hierarchies, brood pheromones, and potentially queen pheromones and other substances that induce stress in these contexts.

It is also possible that there are multiple combinatory signals that are required for reproductive constraint in this species which do not act as a function of stress, but as a redundancy for not accidentally engaging constraint mechanisms when they are not in the interest of the individual. This would contrast with the multiple redundant methods for maintaining reproductive constraint in *A. mellifera*, many of which produce ovary repression. (47)

This latter hypothesis formed the basis of the rationale of the reduced food experiments developed in section 4.3.1.8, and carried out in section 4.3.2.3.4. I.e. that by inducing a greater level of stress from food restriction, we could potentiate the effect of pheromone. This did seem to produce a slight statistically non-significant reduction in ovary activity, and perhaps this experiment could be repeated with greater sample size to investigate the effect of this.

The are potentially other ways of maintaining higher stress in bumblebee workers during experiments as a means to investigate the interaction between stress and activity: increased light exposure was shown to have a significant effect on mortality (Figure 4.6), thus increasing exposure of the cages to light at small amounts; the bees were kept at a consistent 27°C, and so a change in temperature to induce thermal stress; bumblebees are very sensitive to vibration, so consistent vibrational stress e.g. music playing on a speaker attached to the shelving; exposure to carbon dioxide to mimic predator presence; infection with pathogens.

None of these were implemented throughout the course of this thesis due to ethical considerations. The concept of deliberately inducing harm into the bees in order to investigate the possibility of this hypothesis was considered unjustifiable, and certainly not before all other avenues were investigated first, despite the lack of legal regulation to this effect.

4.4.3 *B. terrestris* versus *A. mellifera* reproductive biology

Despite the lack of pheromonally mediated reproductive constraint in these experiments, we do have some evidence to begin to separate the reproductive biology of *B. terrestris* and *A. mellifera*.

In the images shown in Figure 4.2 for the example modified bumblebee Hess scores, the Hess score of 0 was not observed in any cages except for those bees in the starvation experiment. This is a key difference between the *A. mellifera* and *B. terrestris* ovary structure, as in *ad libitum* conditions honeybee workers often exhibit this 0-type morphology.

There likely exists a checkpoint of the *A. mellifera* ovary in the germarium, and reproductive constraint mediated by QMP in this species likely prevents activation via this checkpoint. Additionally, a large proportion of *A. mellifera* workers never exposed to queen pheromones as adults never activate their ovaries, and visible differentiation between the nurse cells and oocytes cannot be observed at the magnifications used for this thesis.

In the *B. terrestris* workers however, some degree of cell differentiation is always visible, with visible yolk deposition in all inactive workers (in the non-starvation experiments). This is reflected in the modified Hess score categories used by other research groups (128, 131) and in this thesis; and how they differ from the categories used for *A. mellifera*. This hints at a fundamental difference in the reproductive anatomy between these two species, which likewise implies a different mechanism of pheromonally mediated reproductive constraint at a molecular level.

This also provides research questions which can be readily answered without the need for QMP-mediated, or nC25-mediated repression to investigate. We can use hybridisation chain reaction (HCR) to investigate the role of e.g. known apoptotic genes, similar to that undertaken by Ronai et al. in *A. mellifera* (257), to determine where in the ovary control is manifested; or other genes associated with reproductive constraint, such as Notch signalling in *A. mellifera* (132). This can be used in bees with active/inactive ovaries from a normal colony context over time as the competition point is reached, to see the location of the molecular origins of reproductive constraint (and make the appropriate comparisons to *A. mellifera* reproductive constraint).

Likewise, it is also possible to do the same observations comparing bees with inactive ovaries due to starvation and bees with inactive ovaries due to reproductive constraint, in order to investigate the difference between these two phenotypes, potentially demonstrating whether reproductive diapause can be eliminated as the manner of reproductive constraint.

4.5 Conclusions

In cavea B. terrestris experiments fail to replicate the reported pheromonally-mediated reproductive constraint of this species demonstrated *in alvo* in the scientific literature.

This could be due to the fact that the previously reported repression is easily obscured by other mechanisms, or only able to function in synergistic effect with other mechanisms.

These other mechanisms might include other pheromones, colony-of-origin genetic factors, age of colony, age of the individual bee, or minimum numbers of bees for social hierarchies.

Despite the lack of reproducibility of these previous findings, we still see indication that the biological basis of reproductive constraint in this species is different than the basis in *A. mellifera*.

4.6 Future Work

4.6.1 Identification of Other Potential Queen Pheromone Compounds

The possibility of other queen pheromone compounds existing, which act synergistically with the identified nC25, or are more appropriate to be named as queen pheromones cannot be ignored. Similarly, the possibility that brood pheromones, or worker compounds acting synergistically with queen products to produce reproductive constraint in workers is a distinct possibility, as it is known to exist in *A. mellifera* (158, 160). Repeating some *in cavea* pheromone experiments with the presence of brood of all ages might be enough to investigate brood effects.

By utilising GC-MS with *B. terrestris* queen washes and gland extracts, as well as comparisons with worker extracts, whilst also performing similar experiments involving brood pheromones from larvae of different ages and different castes (particularly worker and gyne larvae), we can produce a potential library of compounds which can be re-tested individually and in combination in order to investigate the potential synergistic effects of pheromones in this species. (83, 102, 214)

4.6.2 Cumulative Effect of Stress on Reproductive Constraint

Although the ethical considerations of such an experiment would have to be closely considered, the impacts of stressors as a contributing factor to the ability of pheromones to induce reproductive constraint in *B. terrestris* workers could be investigated.

The possible stressors used might be:

- Exposure of the cages to light for a small proportion of each day.
- Increased or decreased temperature of the cages to induce heat/cold stress.
- Introduction of vibration to the cages, e.g. via speakers in contact with the shelving/cages.
- Periodic increases in CO_2 in the vicinity of the cages to imitate predator stress.
- Infection with pathogen to induce immune stress.

Similarly, the food stress experiments conducted in this thesis might simply have to be repeated with larger sample size, and with care and attention to possible colony-of-origin effects. It would possibly also be useful to include an nC25 treatment group, in order to investigate the effect of stressors on this pheromone as well.

4.6.3 Investigation into Differences Between *A. mellifera* and *B. terrestris* Ovaries

In order to investigate the potential structural differences between the two species' method of ovary repression, HCR of known targets of *A. mellifera* reproductive constraint in the ovary could be investigated in *B. terrestris*.

HCR of *in situ* hybridisation has been successfully applied to investigate the difference between somatic and germline cells in *A. mellifera* ovaries (297); as well as the role of apoptosis genes (257), and Notch (and its ligands) in the germline of the *A. mellifera* ovary (132).

This technique could be applied to Notch signalling and insulin signalling genes in bumblebees from different conditions and castes, such as:

- Worker bumblebees with inactive/active ovaries from within pheromonal treatment groups (no treatment, nC25, or QMP) *in cavea*
- Worker bumblebees with inactive/active ovaries from within different starvation response groups *in cavea* (as in section 4.3.1.8)
- Worker bumblebees with inactive/active ovaries *in alvo*
- Wild foraging worker bumblebees
- Queen ovaries *in alvo*

While also producing samples from different age of colony, and age of bee:

- Early founding/first eclosed workers
- Late founding
- Mid-gyne production/pre-competition point
- Post-competition point

By looking at different bumblebees from these different castes and conditions, several questions could be answered:

- Does starvation-induced reproductive constraint match native reproductive constraint, or artificially induced pheromonal reproductive constraint in *B. terrestris* workers?
- Do the *B. terrestris* and *A. mellifera* worker ovary checkpoints work in a similar manner?
- Do bumblebee worker and queen ovary activity exhibit in a similar manner?
- How do these ovary structures change over the lifecycle of a colony?

4.6.4 Methodological Improvements for *in cavea* experiments.

The lack of reproducibility of the effect of nC25 and QMP on *B. terrestris* worker ovary activity, demonstrated in the literature, in this thesis may have simply been due to the, as yet, imperfect development of *in alvo* cage model.

Particularly, larger numbers of workers in each cage, in order to factor in social hierarchy effects on reproductive repression; material out of which the cage is made, to allow easier and more natural movements and possibly more consistent pheromone exposure; and the placement of native comb and brood in each cage at the start of the experimental period, to better simulate a microcolony environment.

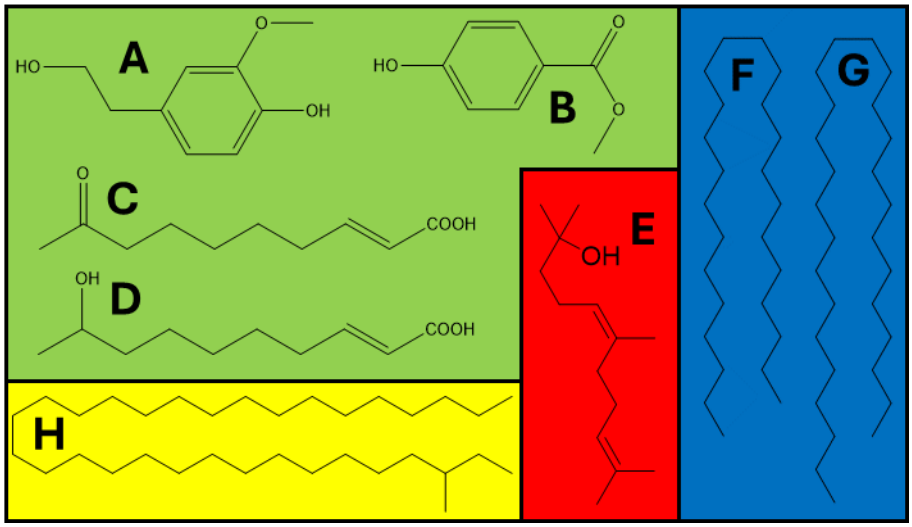
Other methods for investigating ovary activity might also be trialled, such as the use of oocytes per ovary as a testing method. Or indirect methods of observation such as numbers of eggs laid in each cage.

Chapter 5 – How do other pheromones impact insect reproduction?

5.1 Introduction

5.1.1 QMP compared to other Queen Pheromones

Queen pheromones exist widely across eusocial insects, from honeybees to termites. But QMP is unique amongst those discovered for its complexity (46). Most Hymenopteran queen pheromones are simple unbranched or simply branched linear alkanes, barely different (if at all) from the CHCs which make up the anti-desiccants of their cuticles (reviewed in (89)). Termite queen pheromones are slightly more complex, but still usually individual compounds no more complex than nerolidol (shown Box 1**E**) (298). Some other examples of pheromones of different social insects are shown in Box 1; where the relative complexity of the QMP compounds (**A-D**, present in the green box), is very clear when compared to the much simpler long chain hydrocarbons of the *V. vulgaris* wasp and *Cataglyphis* ant (**F** and **G**), *Lasius* species ants (**H**), and the previously mentioned nerolidol of the higher termite *E. neotenicus* (**E**).



Box 1 – Showing queen pheromones from different eusocial insects. **A**-**D** are the chief compounds of QMP, the *Apis mellifera* queen pheromone (108): **A** is homovanillyl alcohol; **B** is methylparaben; **C** is 9-oxo-2-decenoic acid; and **D** represents two compounds, the cis and trans enantiomers of 9-hydroxydec-2-enoic acid . **E** is nerolidol, a queen pheromone for the termite *Embiratermes neotenicus* (298). **F** and **G** are queen pheromones shown to be active in the wasp *Vespula vulgaris* and the ant *Cataglyphis iberica*(46). **H** is a queen pheromone of the ants *Lasius niger* (299) and *Lasius flavus* (300).

Additionally, QMP tends to be far more widely acting than the queen pheromones of other species (such as the fruit fly (148); and bumblebee (209), although the latter of these two was

not reproducible in this thesis *in cavea*). This phylogenetic diversity may be related to its structural complexity via the way that these pheromones evolved. If the workers of honeybee colonies were constantly evolving away from the reproductive constraint of queen pheromones we would expect to see an arms-race style evolution whereby the workers' sensitivity to the compounds would shift, while the nature of the compounds produced by the queen would likewise change with time to maintain that control.

5.1.2 The Evolution of QMP

The snapshot of the evolution of these compounds that we find by observing the nature of QMP in its current form leads us to one of two conclusions: either that this pheromone is still in the process of arms-race style evolution and change, and our snapshot observations are simply a cross-section that would be markedly different in 10s of millions of years time. Or that we observe the end of this arms race and that QMP has managed to coopt a fundamental pathway that cannot now be overcome, and so we would see little-to-no change over time (as with the lack of complexity we can observe of the CHC queen pheromones of *B. terrestris* (46, 88)).

There is evidence from which we can draw either conclusion, which is laid out in Table 10.

5.1.2.1 Snapshot of evolution

For supporting the idea that our view of QMP activity is simply a snapshot of a rapidly evolving pheromone evolution: we see the existence of the anarchic phenotype in beekeeping settings, particularly in the cape honeybee *A. mellifera capensis* (171), though it can spontaneously occur in another similar phenotype in the other strains too (301).

In *A. mellifera capensis* the anarchic phenotype presents as a quasi-parasitic sub-population which exist within hives of otherwise ordinary honeybees of the same subspecies, which have developed the ability to activate their ovaries in the presence of the queen (171). This subspecies of the honeybee also naturally lays clonal diploid eggs via thelytoky (a form of parthenogenesis whereby unfertilised diploid eggs are able to develop into adult females) (171), which are not policed (170) (whereby workers consume the eggs of other workers) in the way they are with other subspecies (129). The *capensis* bee and the devastating effect of its parasitism on the other African honeybee *A. mellifera scutellaris* is a cause of great concern to South African beekeepers due to the economic impact it is having in this industry (302).

Spontaneous desensitisation to QMP (and therefore ovary activation) can also occur in an arrhenotokous manner (whereby workers lay haploid eggs which develop to become drones) in European subspecies (60), though common beekeeping practice is to requeen any colony

which displays large amounts of worker-laid egg presence in colonies (identifiable by multiple eggs being laid per cell, and large amounts of brood comb in sectioned parts of the hive) e.g. as described in reference (303).

The existence of honeybee phenotypes which are able to overcome QMP-mediated reproductive constraint, and can spread this phenotype, implies that these bees are still able to evolve out of this constraint.

This is not a certain conclusion to draw however, as it could easily be argued that this phenotype is an example of a proto-parasitic form, that could evolve with time into a novel obligate parasite of honeybee colonies. I.e. that this particular method of overcoming QMP-mediated reproductive constraint is disruptive enough to the social system of the colony that it fundamentally breaks the eusocial (and so superorganismal) structure of the colony in a way which is an inevitable evolutionary dead-end. I.e. that those honeybee strains which overcome reproductive constraint in this way possess such a high degree of intra-species competition that they are outcompeted by those strains which do not, and eventually become extinct.

5.1.2.2 Completed Evolution

Evidence that we have reached a point-of-no-return with the evolution of QMP comes primarily in two forms: the widespread activity that QMP is able to exhibit; and the role of Notch signalling in QMP-mediated reproductive repression of honeybee workers.

Much study has been done into the wide-acting nature of QMP across the animal kingdom. Queen extract from honeybees, or artificial QMP, has been exposed to, and produced reproductive repression in, a wide array of the animal kingdom: from the *A. mellifera* honeybee (102, 214); to *B. terrestris* bumblebee (209) (~80 million years separated from *A. mellifera* (7)); and the *D. melanogaster* fruit fly (148) (~340 million years separated from *A. mellifera* (3)) in the insects. Indeed, queen extract has also been shown to repress reproduction on a prawn (149), which is separated from honeybees by ~530 million years (304).

The widespread activity of QMP implies that QMP has managed to evolve to coopt a fundamental enough aspect of reproductive repression, that it is no longer able to be evolved away from. This might be linked to diapause via biogenic amine-mediated stress responses (reviewed in (103)), or via notch-mediated paracrine signalling in the ovary (132), or other mechanisms not yet investigated, but ubiquitous across the animal kingdom, or a combination of factors mediated by different elements of the QMP mixture. Notch is particularly well-conserved, and so the discovery of this element of QMP-mediated reproductive constraint in

honeybees implies that this could be acting as an important mechanism underlying the widespread activity of QMP.

However, although QMP is wide-acting, it isn't universal across those tested, such as in the social wasp *Vespula vulgaris* (133). Additionally a meta-analysis by Luke Holman has shown that many different groups' investigations into the effect of QMP have resulted in far different magnitudes of effects, as well as efficacies of different aspects of these compounds (290).

The lack of universality forces us to accept the uncertainty in concluding that QMP has reached a stable composition in its evolution. It is also possible that any further changes to these compounds is driven not by conflict within reproductive constraint, but by the non-reproductive activities of QMP. Components of QMP have been shown to: act as sex pheromones (80); inhibit queen rearing (305); produce queen retinue behaviour (whereby the workers crowd the queen and lick her) (108); attract bees to swarm clusters (91); delay swarming (306); and influences the rate at which workers become foragers (241); Among other roles.

Indeed, natural changes in QMP in relation these other roles may account for the loss of reproductive constraint in the parasitic phenotype of the *capensis* cape bee. i.e. the static nature of the mechanism which underlies reproductive constraint may account for the loss of QMP-mediated reproductive constraint when the mixture of QMP evolves away from this as a result of selection pressures brought about by some other role of QMP. This would result in the composition changing enough that a small subset of bees in a hive which were on the threshold of overcoming this constraint to be able to, and it is this phenotype that we see in the hive of the *capensis* subspecies. However, this would not explain the ability of these bees to parasitise other subspecies.

5.1.2.3 A summary of ideas

The responsiveness of honeybee workers to QMP in many different contexts, across the many different lab groups, in many different parts of the world previously referenced; demonstrates the consistency that different strains and subspecies of honeybee have to QMP. This consistency is not common in the other main class of pheromones present in the honeybee, the brood pheromone (BP), which has a diverse composition and response, even within a subspecies (307).

Strains of honeybee from different locations in USA, with different degrees of interbreeding between the cape honeybee *A. mellifera capensis* subspecies and subspecies derived from European stocks, show very different BP profiles. They also respond to different BP mixtures

with different degrees of sensitivity, when measured by pollen:non-pollen forager ratios, as well as proboscis extension response (measuring sucrose response thresholds). (307)

It is possible that the diversity of these roles of QMP and their importance in eusocial organisation is the driving force behind the evolution of the complexity of QMP. In this scheme the reproductive constraint which QMP has brought about is as a result of QMP being an honest signal of fertility (discussed in general introduction).

Whether QMP is still evolving rapidly, or whether it is now in a stable composition is not a question to which a definitive answer will ever be truly understood, however it is important to consider within the contexts of the other pheromones that exist within this species.

Table 10 – Showing the arguments for whether the complexity of QMP is in ongoing evolution, or whether it is no longer evolving

Evidence for snapshot of ongoing evolution	**Evidence for end-result of arms-race**
Existence of anarchic bees, both the thelytokous *A. mellifera capensis*, and the arrhenotokous other strains (such as the European *A. mellifera ligustica*)	Role of Notch and dopamine signalling as fundamental mechanisms
The wide effects of QMP in non-reproductive constraint roles within the colony	The widespread activity of QMP across different species very distantly related to one another

5.1.3 Other Honeybee Queen Pheromones

Other pheromones produced by the honeybee queen, such as queen's Dufour's gland excretion (92), and tergal gland products, have also been studied, though to a much less rigorous degree. Dufour's gland substance was shown not to induce ovary repression (94), but tergal gland secretions were able to suppress ovary activity in the African bees *A. mellifera scutellaris* and *capensis*(95), though notably tergal glad extract appears to have a high concentration of 9-octadecanoic acid(93), which is very similar to 9-ODA which is present in QMP(102).

Other compounds are also being investigated for their queen pheromone activity in honeybees, such as the CHC mixtures investigated by Princen et al (47). This group found that artificial mixtures of CHC compounds were able to suppress ovary activity in workers at least as well as QMP, and found a large deal of redundancy between the different elements of these compounds and their ability to suppress worker reproduction.

Extracts from honeybee larvae, particularly BP and E-β-ocimene (EBO), have also been shown to suppress ovary activation in *A. mellifera* workers (158, 160).

5.1.4 Honeybee Larval Pheromones

Brood pheromone is a mixture of methyl and ethyl fatty acid esters (summarised in Table 11), produced by the developing larvae (159). Honeybees spend five days in larval development, across 6 instars (49) shown in Figure 5.1. The proportion of each compound present in this mixture, and the total amount produced is different at different ages, with the most BP being exuded just before cell-capping on day 8 (shown in Figure 5.1).(159)

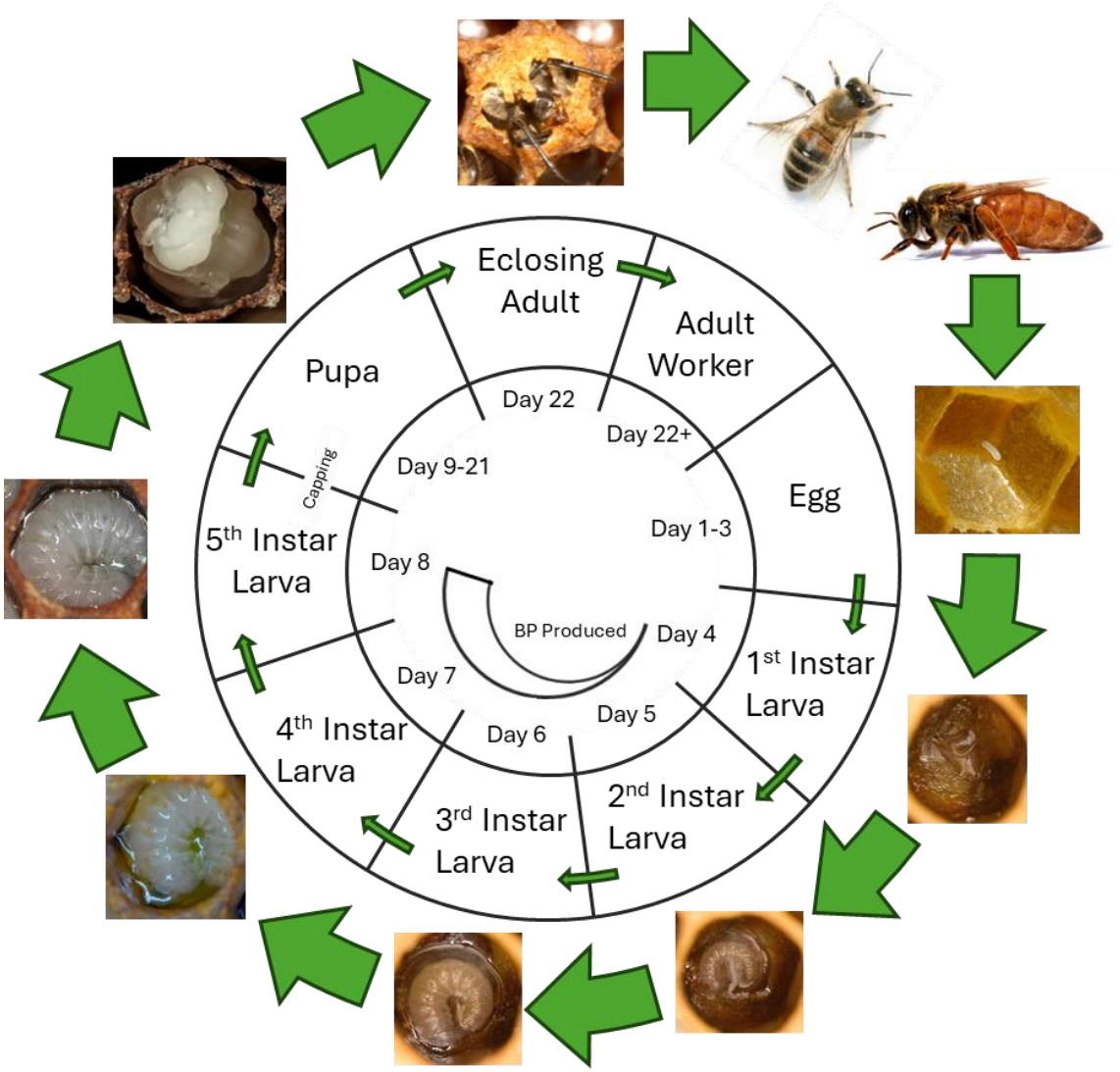


Figure 5.1 – Showing the lifecycle of an *Apis mellifera* worker, focusing on the larval stages. The egg is laid by a queen, and after three days hatches a first instar larvae. The larva moults once a day until day 8 when the cell is capped and the prepupa undergoes metamorphosis. After another 12 days an adult ecloses. Over its five larval instars, it produces an increasing amount of BP (22, 159, 308-312)

The role of BP is fairly diverse, with different elements of BP producing both primer and release responses with different effects on adult workers in the hive. BP has been shown: to influence the ratio of pollen foragers to nectar foragers, and total number of foraging flights (163); increase the number of total foragers (44); increase the average pollen load (164); decrease worker longevity (157); and recruit for feeding and cleaning behaviours in nurses (313). Components of BP have also been shown to induce capping behaviour, and influence queen development in *Apis cerana* and *A. mellifera* (whereby manual rearing of queens by hand *in vitro* with royal jelly supplemented with differing concentrations of BP components; showing that increased methyl palmitate and ethyl oleate concentrations increased likelihood of successful capping, and increased methyl palmitate concentrations increased larval weight gain). (314).

EBO is also produced by the developing brood and has also been shown to have repressive effects on the development of honeybee ovaries (160). As with BP, this effect is not its only role within the colony: it has also been shown to induce earlier foraging behaviour (160); and to recruit nurses for feeding (161).

While both BP and EBO have been established as pheromones responsible for many roles within colonies in a number of different publications, there is a dearth of publications in which reproductive constraint has been demonstrated, and only by a single lab group (158, 160, 166).

This lab group demonstrated that BP as a mixture is able to repress worker reproduction when administered via contact, diffusion, and ingestion (315), and that the components methyl linolenate and ethyl palmitate are principally responsible for this repression (158), using an *in cavea* model similar to that used in this thesis. They also showed that there is a minimum concentration of these components which produces reproductive repression (between 62 and 620 Leqs of BP components), with higher concentrations up to 6200 Leqs producing greater effect sizes of repression. In addition to these findings, it was also noted that the components likely act synergistically, as the effect size on reproductive repression when components are combined appears to be larger than the additive repressive effects (158).

There is also a lack of information as to the mechanism of action by which these pheromones might induce this reproductive constraint. As a result, there is an opportunity first to reproduce these findings, and then to continue on to begin to investigate the mechanism of action of these pheromones at a molecular level.

5.1.5 Alternative Animal Models

There are several possible target organisms for investigating the effect of pheromones which induce reproductive constraint in honeybee workers. The wide array of species across which QMP is able to act, gives us many possible outgroups. Particularly the bumblebee *B. terrestris*, as investigated in Chapter 4, and the fruit fly *D. melanogaster*.

5.1.5.1 D. melanogaster

The fruit fly *D. melanogaster*, which has been demonstrated to respond to QMP by several lab groups (134, 148, 316), provides us with a particularly powerful outgroup. The diptera and hymenoptera orders are separated by some 340 million years (3), and so the ability of high concentrations of QMP to inhibit reproduction in this organism is rather unusual. Whether this is because honeybee reproductive pheromones are able to act widely, or whether QMP is unique is an important question to be answered, and this organism provides us with a very powerful tool to do so.

D. melanogaster has been used as a model organism for developmental biology since the very early 20th century (317), as it is incredibly easy to rear in an artificial context, and with very short generation times of 10 days at optimal growth conditions, as well as a very consistent development time for each stage of larval development (317). Many very useful tools have also been developed to manipulate the genetics of this organism, particularly via the use of transposon elements (318), balancer chromosomes (319), and the UAS-Gal4 system (320). The common use of *D. melanogaster* also means that there are large repositories of mutants easily obtainable and with little fuss (e.g. from the Bloomington stock center in Indiana, US; or from the Vienna Biocenter), as well as a great deal of support in their management, especially within institutions.

The amount of understanding we have of the underlying biology of the fruit fly, as well as these extensive tools, allow us to investigate the effect of QMP in this organism to a much higher degree of precision than we are able to in honeybees.

With regards to its use in pheromone exposure assays, this has also been readily established (e.g. (134)), and so the setup time of this organism in a new lab context is very low, with very short assay lengths, and relatively simple dissections and analysis.

Lovegrove et al. (134) demonstrated that the bumblebee queen pheromone pentacosane (nC25) was not able to repress reproduction in this animal, despite the ability of QMP to. This has been disputed however by a paper accessible on BioRXiv (135), though this paper seems not to have made it to publication (N.B. as of 23/04/25 this paper has been taken down from

BioRXiv and replaced with a newer version (321), though the relevant information pertaining to this thesis is the same across both versions).

Velasque et al. purport to show that nC25, as well as several other pheromones such as honeybee BP, termite queen pheromone n-butyl-n-butyrate and 2-methyl-1-butanol, as well as a QMP and BP mixture, all produced significant repressive effect on fly reproduction. In order to show this, they mix the pheromones into the media on which the flies are reared. Adult CantonS flies are allowed to lay on the treated food, and the eggs are allowed to develop into adults on the same food, virgins collected and kept on the same food media, before being dissected and ovaries imaged before being measured by observing the area of each ovary in each image.

There are several issues with this experimental protocol, but the greatest is the concentrations of pheromones which are being used. In this experiment, 10 ml of media is provided in each vial of flies, into which has been mixed 67 µl of pheromone mixture. The media therefore contains a 1:150 dilution of pheromone mixture, while the pheromone mixtures have already been diluted: for QMP this represents 0.017Qe/vial; for nC25, $2.87x10^{-5}$ Qe/vial; and for BP, 20 Leq/vial. These concentrations are vanishingly small compared to the amounts of pheromones which have been tested in other contexts e.g. Camiletti et al. used a minimum of 20 Qe of QMP/vial, as an aliquot in direct contact with the adults (148). Similarly, Lovegrove et al. demonstrated no effect of nC25 on adult fruit fly reproduction at 26 Qe of pheromone per vial in the same exposure as Camiletti et al. (134, 148, 265). Again, similarly, the concentration used to demonstrate BP-mediated reproductive repression in honeybees was at a concentration of 620 Leqs of BP applied to honeybee workers (158).

The concentrations used in this experiment are therefore in the order of 1000x more dilute for QMP; 90,000x more dilute for nC25; and 31x more dilute for BP (compared to honeybees).

The difference in findings between these setups, might be a consequence of the biological environment and nature of exposure (exposure in media from egg-adult vs in food as adult), and so this requires a third testing to attempt to reproduce the findings presented therein.

5.1.6 Quercetin

Gao et al. (322), demonstrated that the plant polyphenol quercetin was able to prevent some of the QMP-mediated colony-wide effects of QMP activity in honeybees. Plants produce polyphenols under conditions of stress, including chemical and temperature (reviewed in (323), likely as anti-oxidants. These polyphenols build up in tissue, including the nectaries and nectar of the plant, whence pollinators interact with them.

This finding then has serious implications for the impact of climate change on eusocial pollinators such as honeybees. As we see increases in the temperature stress brought about by climate change, this may result in increasing lack of sensitivity to QMP in an in-hive setting.

However, this paper has several methodological flaws. These included the way the bees were mixed before the experiment began, which can often result in intense intracolony competition consistent with the observed effects for inhibited queen pheromone activity (greater worker egg-laying, queen cell production, aggressiveness of workers). The numbers of different colonies used for this experiment was limited to three colonies per treatment, which is very low, additionally the number of bees per colony was also very low at only 10,000 workers. The amount of quercetin fed to each colony was at the limit of what is able to be dissolved in the sugar liquid used to administer, which the research group claims is biologically relevant, but given the lack of solubility of this compound seems suspect. The bees were also given very little choice in food availability, and were not allowed to forage widely, limiting the usefulness of using whole-colony analysis.

Despite the flaws in this paper, the conclusions drawn potentially demonstrate a high degree of impact, and so it was worth checking this proposed interaction between quercetin and QMP in the *D. melanogaster* model, as well as in our *A. mellifera in cavea* model, where we are able to isolate the biological facet being investigated in a way which is less feasible *in alvo*.

5.1.7 Research Questions

The research herein will attempt to answer the questions:

- Can the published repressive effect of BP and EBO on worker ovaries, be reproduced in our *in cavea* honeybee setup? And if so, are the mechanism through which BP and EBO able to bring about this repression the same as QMP in honeybees?
- Is QMP unique amongst the honeybee pheromones for its broad phylogenetic activity in ovary repression? Can BP and EBO inhibit the ovary activation of *D. melanogaster* fruit flies?
- Can the findings that plant polyphenol quercetin is able to inhibit QMP activity in honeybees be reproduced giving us a chemical QMP inhibitor for future experimental design? And is it able to inhibit the activity of QMP in *D. melanogaster* demonstrating similar mechanisms of action of QMP between honeybees and fruit flies?

5.2 Methods

5.2.1 Pheromones

5.2.1.1 Brood Pheromone

Brood Pheromone (BP) is a mixture of 10 fatty acid esters laid out in Table 11. The composition was adapted from (159). The compounds are waxes and oils dissolved in ethanol and stored as a 10x stock at -20°C. The working 1x dose is 617Leq (with one Leq being the amount produced in a single day by an 8 day-old larva (159)). This is dissolved in ethanol at 617 Leq /20 µl and applied according to experiment.

Table 11 – Showing the relative composition of Brood Pheromone and suppliers

Component	Proportion	µg required for 617Leq	Supplier
EL: ethyl linoleate	1	10	Sigma
ELN: ethyl linolenate	11	110	Sigma
EO: ethyl oleate	15	150	Aldrich
ES: ethyl stearate	13	30	Sigma
EP: ethyl palmitate	7	10	Alfa Aesar
ML: methyl linoleate	1	200	Chem Cruz
MLN: methyl linolenate	23	130	Fluorochem
MO: methyl oleate	20	230	Acros Organics
MS: methyl stearate	6	70	Alfa Aesar
MP: methyl palmitate	3	60	Sigma

This concentration of BP was chosen as it closely resembles the concentration of Mohammedi et al. (158) and Le Conte et al. (324), who supplied the BP and individual components of BP individually at a concentration of 1mg/g of fondant, which they estimate to be 620 Leqs per day.

Additionally, 26 Qe of QMP (used throughout this thesis as the maximum QMP concentration applied) equates to 9.932 mg of material, which equates to 6158 Leqs of BP if comparing raw masses of compounds. As a result, a 10x stock of BP was produced with 10 mg of BP producing 6170 Leqs/20 µl of ethanol, which was diluted to 617 Leqs as a standard. This falls within the middle of the range of BP concentrations used by different lab groups for investigating the effects of BP (155, 158, 324, 325).

5.2.1.2 MLNEP

Mohammedi et al. also demonstrated that increased concentrations of BP components produced a greater effect size of reproductive repression (158). As a result, a simpler blend of BP containing the two components MLN and EP at concentrations of 1625 µg/20 µl and 17.2 µg / 20 µl respectively. This 10x stock was diluted to 100Leq/ 20 µl and 600Leq / 20 µl respectively for a working stock. This represented the upper limit of these pheromones which was soluble in ethanol. This was to investigate the effect of higher concentrations of these components on ovary activity in worker honeybees.

5.2.1.3 Ocimene

E-β-ocimene (EBO)(Ocimene, Sigma-Aldrich) was dissolved in ethanol to produce a 10x stock of 495.5 mg/ml, equivalent to 11,100 Leq (162). Stored at -20°C. 1,110 was chosen as the 1x stock, as it closely resembles the concentration used by Maisonnasse et al. to demonstrate EBO's ability to repress reproduction in honeybee workers (160).

5.2.1.4 Quercetin

Quercetin (Fluorochem) was reported to be dissolved in sugar water at a concentration of 0.005% (w/w) (326), however this is far over the capacity of water to dissolve quercetin. As a result, the quercetin was dissolved in DMSO at a comparable concentration of 0.005% (w/v) (322), which equates to 0.16 mM quercetin in DMSO.

5.2.2 Honeybee Cage Assays

All honeybee cage assays were as laid out in section 2.4.2.1, with the exception of the cages presented in Figure 5.3**E** in which the cages had also been enclosed within a 28 cm x 26 cm x 20 cm plastic box (Watkins and Doncaster) shown in Figure 5.2. This was to limit the diffusion of pheromone away from the cage.

In Mohammedi et al. (158), they provided 120 bees per cage with water *ad libitum* as here, however they provided food as pollen and fondant. They also provided comb, making sure to destroy any eggs laid during the course of experiments, as well as applying treatment not via

microscope slide as is standard in our *in cavea* setup, but mixed into the fondant, at a concentration that they estimate to be exposing the bees to the equivalent of 620 Leq per day (324, 325).

Figure 5.2 – Showing a honeybee cage contained within a plastic box to limit dissipation of pheromone away from cage

The honeybee cage assays were performed during different parts of the summer beekeeping season (from May to September) across 2 years: 2022 and 2023.

The different figure panels and the years they were carried out in are shown in Table 12.

Table 12 – Showing the year in which each honeybee cage assay experiment was carried out**.**

Summer 2022	**Summer 2023**
Figure 5.3**A**, Figure 5.3**B**, All of Figure 5.4	Figure 5.3**C**, Figure 5.3**D**, Figure 5.3**E**

The experiments of the summer 2022 were carried out on a CBF diet, with the exception of the experiment of Figure 5.4**B.** This figure and experiment and all other *in cavea* experiments in this chapter were performed using the FandP diet (see section 2.2.3.1 for details of diets), in order to more closely align with the protocol of Mohammedi et al. (158).

Pheromone treatment was applied as detailed in 2.4.2.1, with QMP, BP, MLNEP, and EBO being applied as a 20 µl aliquot onto a microscope slide placed in the bottom of the cage, refreshed daily. With the exception of the experiment shown in Figure 5.3**C**, which had the BP administered as a topical application to the CBF food which was being fed to the bees, this was to align more closely to the protocol of Mohammedi et al. (158), in order to test if the manner of application of pheromone was essential for BP reproductive repression (though this is implied not to be the case by Arnold et al. (315), who showed that contact was sufficient to bring about repression of worker reproduction via BP).

5.2.2.1 Quercetin exposure

Quercetin exposure was carried out in the same manner as the caged experiments in the previous section, as according to sections 2.4.2.1, 2.5.1, and 2.6.1.1. With the exception that as quercetin was dissolved in DMSO, the QMP and Ethanol controls also contained DMSO as a solvent control.

5.2.3 Fruit Fly assays

All fly assays were carried out as laid out section 2.4.1, with identical virgin collection and 2-day assay protocol. Except for the data shown in section 5.3.2.3.

26 Qe of QMP was chosen as the maximal concentration for QMP exposure as it is the concentration used by Lovegrove et al. (134). The BP and EBO concentrations are used as they match the concentrations used in the honeybee experiments.

5.2.3.1 Velasque et al repeat

Bloomington fly food (see section 2.2.3.2.1) was made up and additives of pheromone were added in the manner of Velasque et al (135): food was made up with 50 µl pheromone added at liquid stage into 150ml of fly media, aliquoted into 15 x 10 ml vials, control solvent was also added to each (e.g. the QMP vials contained the QMP and solvent, but also 50 µl of heptane, as a control for the solvent of nC25). Pheromones detailed in Table 13.

To five of each vial five males and five virgin female OregonR wild-type flies were placed and allowed to mate and lay eggs for 72h at 25°C. The adults were removed and the vials were kept at 25°C until F_1 adults eclosed. Virgins were collected from these vials and placed onto fresh vials for 6 days then dissected according to section 2.5.2 and fixed according to section 2.6.2.1.

After fixing, ovaries were imaged at 15x magnification and their length was recorded using Fiji-ImageJ, by measuring the number of pixels lengthwise along the ovary.

The differences between the Velasque et al. paper and this experimental protocol, is the wild-type strain of fly, which here is OregonR versus the CantonS used in this paper; the manner of ovary measurement, which here is given as ovary length rather than ovary area; and the concentrations of pheromone, which were 6.5 Qe QMP, 617 Leqs BP, and 1 Qe nC25 in each respective vial.

A second experiment was undertaken investigating the effect of strain, shown in Figure 5.10, using the fly vial method of exposure detailed in sections 2.4.1.2, 2.5.2, 2.6.2.1, and 2.6.2.2.

Pheromone	Amount used	Final concentration per 150 ml media
QMP	1Qe into 50 µl ethanol	0.02 Qe
Brood Pheromone Commercial (BPT)	20 µl raw pheromone into 30 µl ethanol	Unknown original conc.
Brood Pheromone mixture (BPO)	10 µl 6170Leq mixture into 40 µl ethanol	1234 Leqs
nC25	1 µg into 50 µl heptane	0.02 µg nC25
Ethanol control	50 µl ethanol	/
Heptane Control	50 µl heptane	/

Table 13 – Showing the amounts of pheromone in each additive applied to Bloomington fly media for Velasque et al. experiment.

5.2.4 Statistics

5.2.4.1 Honeybee experiments

Ovary score data was processed and analysed as detailed in section 2.6.1.1.

Mortality data and Kaplan-Meier analysis was carried out as detailed in section 2.6.1.3

5.2.4.2 Fruit Fly experiments

Ovary activity analysis was carried out as detailed in section 2.6.2.2, except for the data presented in Figure 5.9, which was measured as continuous data, and so a continuous Cullen and Fray graph was used instead, with appropriate differences in distribution analysis.

5.3 Results

5.3.1 Honeybee Brood Pheromone and E-β-Ocimene

In *A. mellifera* two different types of larval-pheromones have been indicated as having effects on insect reproduction: Brood Pheromone (BP) (158), and E-β-Ocimene (160).

In order to investigate the mechanism of action of these pheromones, these effects had to be reproduced in our *in cavea* setup.

5.3.1.1 Brood Pheromone does not prevent ovary activation in *A. mellifera* worker ovaries in our *in cavea* setup.

Shown in Figure 5.3**A**, a mixture of BP derived from references (152, 154, 158, 324, 327, 328) failed to produce any degree of ovary repression (CLMM: $\chi^2 = 18.87$, df= 2, P<0.001), as did a repeat with freshly purchased BP components shown in Figure 5.3**B** (CLMM: $\chi^2 = 11.817$, df= 2, P=0.01). In both of these tests the BP treatment group was indistinguishable from the ethanol control (results of post-hoc pairwise tests in Table 49 and Table 50, in the appendices). Both of these experiments were carried out with the bees being fed CBF.

The use of the CBF diet was in line with previous research (132), but is less biologically relevant than the FandP diet (detailed in section 2.2.3.1.2), which allows for a choice between the natural food types (as discussed in 3.3.1).

Figure 5.3 **C**-**D** show bees being fed on the more biologically relevant FandP diet, and exposed to treatment in different ways, however this still failed to produce any statistically significant efficacy of BP.

In **C**, the bees were exposed to treatment in the food (in order to investigate whether mode of exposure was important for inducing BP effect). However, in this experiment biological repeat (CLMM: $\chi^2 = 5.7835$, df = 1, P = 0.0162) showed a greater effect on ovary score than treatment (CLMM: $\chi^2 = 1.4232$, df= 2, P=0.49). The lack of effect of treatment may possibly have been due to sublethal toxicity effects of the ethanol dissolved in the food (all pairwise comparisons shown in Table 51, in the appendices).

In Figure 5.3**D**, where the bees were exposed to BP on a microscope slide on the bottom of the cage (as in Panels **A** and **B**) (CLMM: $\chi^2 = 33.502$, df= 2, P>0.0001), there was no statistically significant difference between BP and the ethanol control, but there was between BP and the QMP sample (results of pairwise tests in Table 52, in the appendices).

The volatility of BP may have been responsible for its lack of activity, and so in **E** the cages of bees were sealed in plastic boxes inside the incubator to limit diffusion of the pheromone away (CLMM: χ^2= 6.695, df= 2 , P=0.035). However, this seemed to prevent the QMP from being able to suppress the ovaries of workers, and so there is no statistically significant difference between any of the samples (results of pairwise tests in Table 53, in the appendices). This lack of repression is possibly as a result of humidity effects, but it is also possible that the enclosed space prevented the diffusion of QMP components away from the cage such that the local concentration was much higher, and it has been shown that QMP is unable to repress workers at higher concentrations (104).

The failure of BP to induce ovary repression in any of these three experiments investigating the method of application of BP shows that this is not the important factor explaining the lack of reproducibility of Mohammedi et al. (158) and Arnold et al. (166), nor is biological relevance of food type the explanatory factor. Mohammedi et al. (158) implicated methyl linolenate (MLN) and ethyl palmitate (EP) as the two substances in BP most responsible for ovary repression, by using these two substances alone, we were able to increase the concentration of BP in order to investigate if the effects reported by Mohammedi required a higher concentration than we had previously exposed the bees to.

These results are shown in Figure 5.4**C**-**E**. In **C**, bees fed on CBF diet and exposed to MLNEP (CLMM: χ^2= 18.897, df= 2, P<0.001) showed no significant ovary repression compared to ethanol, but significant difference in expression to the QMP group (results of pairwise tests in Table 56, in the appendices). **D** shows bees exposed to MLNEP and also fed on the FandP diet, but is almost identical in both trend and result to panel **C** (CLMM: χ^2=16.005, df= 2, P<0.001; results of pairwise tests in Table 57, in the appendices). These results demonstrate that the concentration of BP being too low to instigate effect was not the explanatory factor behind the lack of reproducibility of BP's repressive ability on ovary activation in honeybees.

In panel **E** we see bees exposed to a mixture of QMP and MLNEP, in order to investigate whether QMP presence is necessary to result in further repression of worker ovaries, i.e. whether the effects of BP are synergistic with QMP (CLMM: χ^2= 19.228, df= 2, P<0.001). In this case we see no statistically significant difference between MLNEP+QMP and the QMP control, and we do see difference between the Ethanol control and the treatment mixture. However, we also see that while not statistically significant, there does seem to be some mitigation of the suppressive effects of QMP by the presence of MLNEP. (results of pairwise test in Table 58, in the appendices)

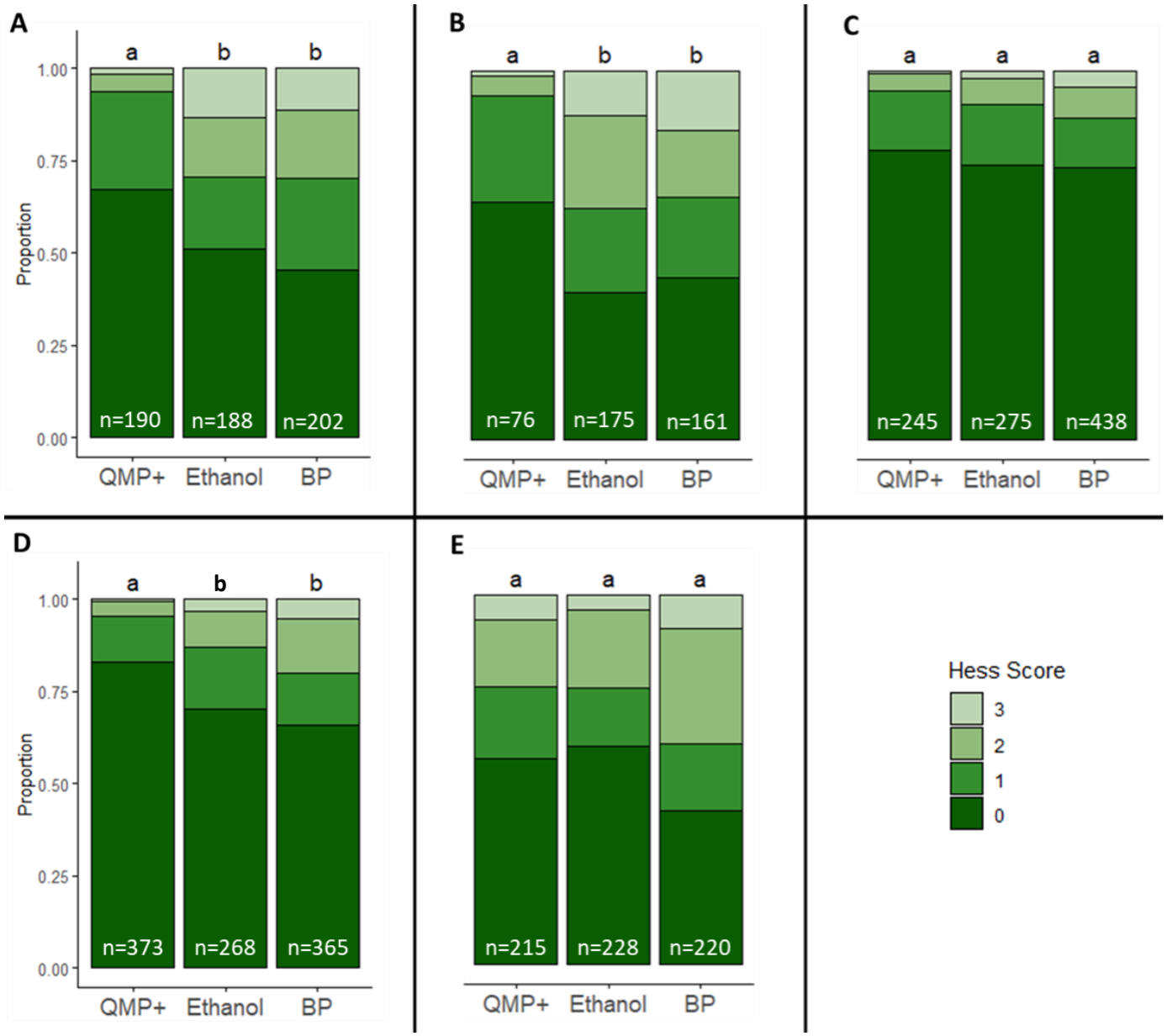


Figure 5.3 – Showing the response of *A. mellifera* worker ovary development to Brood Pheromone.

The y-axis is the proportion of ovaries with Hess scores of 0-3 (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present) of *Apis mellifera* honeybee workers in exposure to different pheromonal treatments shown on the x -axis. In all panels (**A**-**E**), "QMP+" represents bees exposed to 0.1Qe QMP, "Ethanol" represents bees exposed to solvent control, and "BP" represents bees exposed to 617Leqs *A. mellifera* Brood Pheromone (BP). **A** is the first BP mixture attempt from the Summer of 2022. **B** shows a freshly produced BP mixture from the same Summer. **C** is of bees which were exposed to the Treatments in the food from Summer 2023. **D** is a repeat of BP on slide from Summer 2023. **E** shows results of bees whose cages had been enclosed within plastic boxes inside the incubators from Summer 2023. Both **A** and **B** were bees fed CBF diet, while **C**, **D**, and **E**, were bees fed using the FandP diet. In all cases, Treatment failed to produce suppression of ovary activation. In all cases , the n-value for each treatment is shown in each bar, and statistical significance is given as letters $P<0.05$, calculated via CLMM and post-hoc Tukey pairwise test.

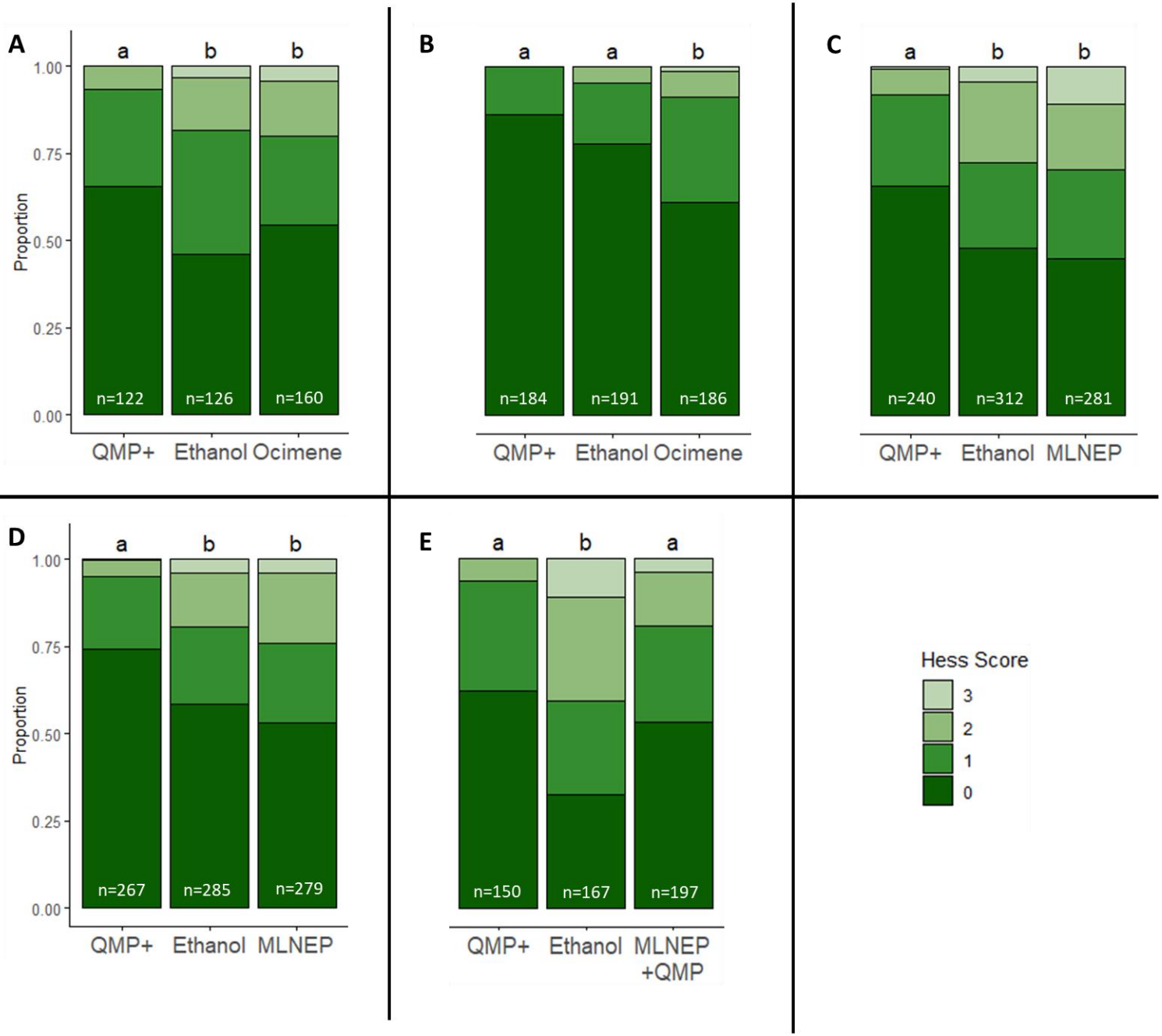


Figure 5.4 – Showing the response of *A. mellifera* worker ovary development in response to Ocimene and MLNEP.

The y-axis shows the proportion of ovaries with Hess scores of 0-3 (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present) of *Apis mellifera* honeybee workers in exposure to different pheromonal treatments shown on the x -axis. In all panels (A-E), "QMP+" represents bees exposed to 0.1Qe QMP and "Ethanol" represents bees exposed to solvent control. A shows bees exposed to an ocimene mixture on a CBF diet from Summer 2022. B shows bees exposed to and ocimene mixture on an FandP diet from Summer 2022. C shows bees exposed to the MLNEP mixture on CBF diet from Summer 2022. D shows bees exposed to the MLNEP mixture on FandP diet from Summer 2022. E shows bees exposed to the MLNEP mixture as well as QMP, fed on a CBF diet from Summer 2022. In all cases treatment failed to prevent ovary activation. In all cases , the n-value for each treatment is shown in each bar, and statistical significance is given as letters $P<0.05$, calculated via CLMM and post-hoc Tukey pairwise test.

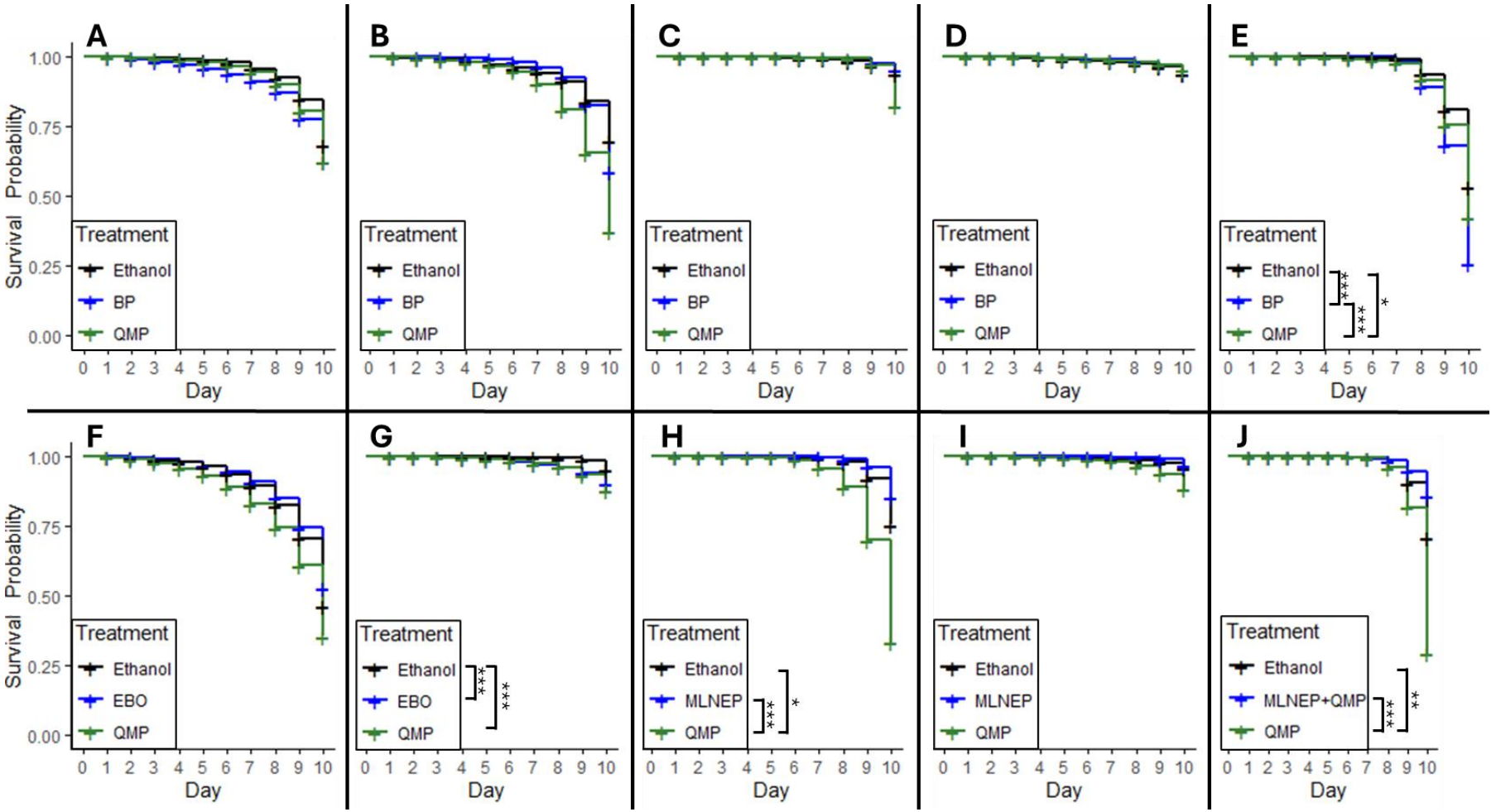


Figure 5.5 – Showing Kaplan-Meier curves for each of the honeybee experiments in this chapter. The y-axis shows the probability of survival for a given individual, the x-axis shows the day of experiment. **A** shows the First BP exposure in the Summer of 2022 with CBF diet. **B** shows the second BP exposure from the Summer of 2022 with CBF. **C** shows BP exposure in food from Summer 2023 with FandP diet. **D** shows BP exposure on microscope slide from Summer 2023 with FandP diet. **E** shows bees exposed to BP in cages contained within plastic boxes from Summer 2023 with FandP diet. **F** shows EBO exposure from Summer 2022 with CBF diet. **G** shows EBO exposure from Summer 2022 with FandP diet. **H** shows MLNEP exposure from Summer 2022 with CBF diet. **I** shows MLNEP exposure from Summer 2022 with FandP det. **J** shows MLNEP mixed with QMP exposure from Summer 2022 with CBF diet. Significances given as: *= P <0.05, **= P<0.01, ***=P<0.001 and were calculated by CoxME model and post-hoc pairwise Tukey test.

Therefore, at no point, in our setup, could BP be shown to have suppressive effects on ovary activation in *A. mellifera* workers.

5.3.1.2 E-β-Ocimene also produces no inhibition of *A. mellifera* ovary activation

As shown in Figure 5.4**A** (CLMM: χ^2= 11.24, df= 2, P<0.001), the racemic mixture of Ocimene (E/Z – β – ocimene) was unable to produce repression of ovaries. The ocimene treatment group was very similar to the ethanol control and significantly different from the QMP bees (results of pairwise test in Table 54, in the appendices).

As the experiment in panel **A** was carried out under a CBF diet, it is possible that diet affected the ability for EBO to produce repressive effects, and so **B** shows the same ocimene exposure as in **A** but fed on FandP diet. The controls did not differ from one another, and there is a statistically significant activation of bees exposed to ocimene compared to both QMP, and Ethanol (results of pairwise tests in Table 55, in the appendices). It is notable that in the CLMM produced for the data contained in panel **B**, the model was over-converged as a result of a lack of ovary score 2s and 3s. As a result the statistical analysis for this graph is less reliable than the other graphs. Multiple different models were attempted and all showed over-convergence, and so the statistical analysis data represents the best fitted model, rather than a well-fitted model.

That the data shown in **B** showed no difference in reproductive repression between the two controls can possibly be explained by the fact that this experiment was carried out in September 2022, where the winter-bee phenotype (the long-lived winter-specialised worker type) had started to be exhibited. The winter phenotype is thought to be caused by diminishing pollen availability resulting in diminishing brood development (329). This would also then explain the significant activation of the ocimene group, as ocimene would partially inhibit the winter phenotype by mimicking the presence of brood.

5.3.1.3 Brood Pheromone, MLNEP, and E-β-Ocimene did not show consistent effects on mortality

As can be seen in Figure 5.5, there is broadly no consistent effect on mortality. In all cases, even where significance is shown, the “cage” variable showed greater effect on survival probability than treatment.

Additionally, Figure 5.6**A** shows the impact of BP on survival rates across all experiments, showing no significant impact of BP on survival. While Figure 5.6**B** shows the same result of aggregated E-β-ocimene treatment.

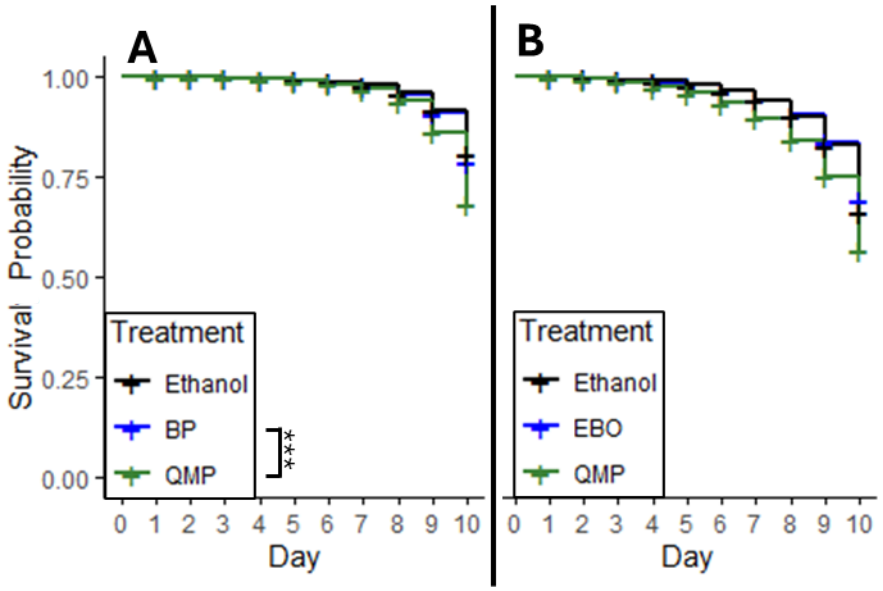


Figure 5.6 – Showing Kaplan-Meier curves for the data of Figure 5.5 aggregated by treatment. The y-axis shows the probability of survival for a given individual, the x-axis shows the day of experiment. **A** shows the aggregate of all BP experiments and MLNEP experiments across Summer 2022 and 2023, with mixed diets. **B** shows the aggregation of the two ocimene experiments of Summer 2022 with mixed diets. Significance shown as ***=P<0.001

5.3.2 *D. Melanogaster*

It has been consistently shown that the female of the fruit fly *D. melanogaster* is able to be reproductively constrained in the presence of QMP (148, 183). This is one example of many of the broad phylogenetic effects of QMP (290).

The efficacy of QMP in this fruit fly could be an example of convergent evolution towards the same biological mechanism being used for reproductive repression in the two organisms; ancestral effects of e.g. Notch signalling being taken advantage of by the honeybee queen to instigate ovary repression by a different sensory stimulus, but the same mechanism; or incidental effects, whereby the ability of QMP to act in *D. melanogaster* is simply the result of chance interactions.

The ease of use of the fruit fly as a model makes it ideal to test the broad phylogenetic effects of other *A. mellifera* pheromones, as in (134).

5.3.2.1 QMP-mediated ovary repression in *D. melanogaster* acts in a dose-dependent manner

First, the effectiveness of our QMP stock on this strain of OregonR wild-type fly was tested (Figure 5.7), both to evaluate the efficacy of this QMP stock, but also to validate the protocol in

our lab environment. Using an exponentially increasing concentration of QMP, from 3.25 to 26 Qe, the flies showed a statistically significant proportional decrease in ovary activation when measuring the number of stage 14 vitellogenic oocytes per ovary, similar to that previously reported in the literature (148, 183, 216, 316) (GLM: df = 359, F = 61.154, P < 0.001), results of pairwise Tukey tests shown in Table 59 in the appendices.

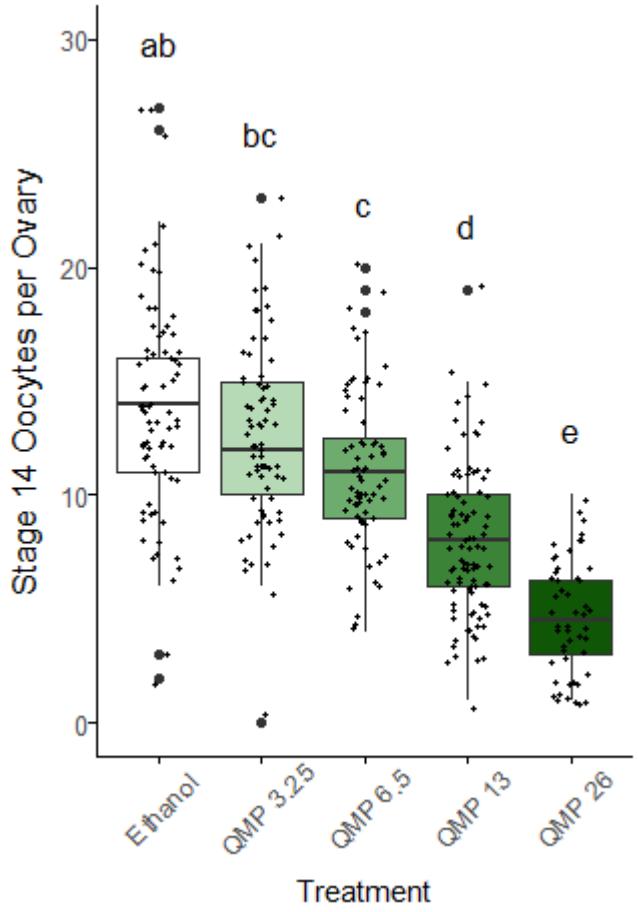


Figure 5.7 – Showing the QMP dose-response for wild-type OregonR flies. The y-axis shows the number of stage 14 vitellogenic oocytes per ovary, and the x-axis shows different treatment groups in increasing concentration of Qe of QMP, with ethanol as a 0 Qe QMP control. The significances are shown as letters, P<0.05, calculated via GLM and post-hoc pairwise Tukey tests. There is a clear statistically significant trend of increasing QMP concentration results in decreasing ovary activation (n = 363).

5.3.2.2 Neither BP nor E-β-ocimene produce any ovary repression in *D. melanogaster*

Having validated the QMP, the two pheromones produced by the developing larvae of the *A. mellifera* honeybee, tested previously in honeybee workers were tested in their efficacy against *Dm*: EBO, BP; as shown in Figure 5.8. In both pheromones there is a clear lack of repression

brought about by the exposure to these pheromones (GLM_{EBO}: df = 365, χ^2 = 70.126, P < 0.001; GLM_{BP}: df = 525, F = 30.817, P <0.001). Results of individual pairwise comparisons given in Table 60 and Table 61 in the appendices.

At higher pheromone concentrations (11,100 Leqs EBO, and 6170 Leqs BP), there was also high mortality. In the 11,100 EBO treatment group mortality was at 100%, whereas in 6170 BP treatment group, mortality was at 55% across repeats: This presumably accounts for the high zero-weighting of this sample, and that the induced repression is toxicity related.

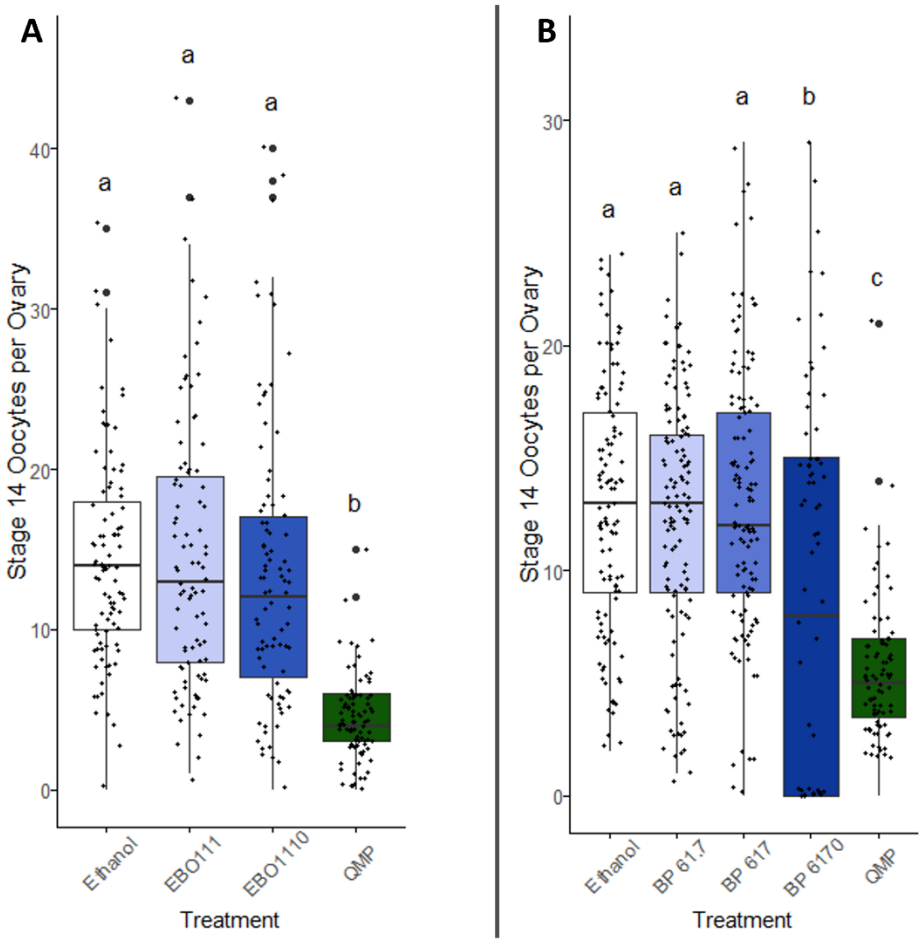


Figure 5.8 – Showing the response of wild-type OregonR flies to two types of pheromone produced by developing *A. mellifera* worker larvae A: EBO (E-β-Ocimene), and **B**: BP (Brood Pheromone). The y-axis shows number of stage 14 vitellogenic oocytes per ovary, and the x-axis shows the different treatments and concentrations of pheromone: Ethanol being the negative control, QMP positive control, and intermediate different concentrations of larval pheromone. Treatment concentrations are given as Larval equivalents (Leqs: the amount produced by a single larva in a day). Significances are given as letters where P < 0.05, calculated via GLM and post-hoc pairwise Tukey tests (**A**: n = 368; **B**: n = 530).

5.3.2.3 Response to Velasque et al.

A paper in review on BioRXiv by Velasque et al. (135), suggested that the effects of the pheromones BP and Pentacosane (shown not to be effective in *Dm* by Lovegrove et al. (134)) were repressive towards *Dm* ovaries. Their protocol was repeated in our lab setting to check for reproducibility, the results of which can be seen in Figure 5.9 and Figure 5.10. Figure 5.9 represents results using a copy of their methodology verbatim (though with a different strain of Wild-type fly, CantonS), using the concentrations and methods of exposure which Velasque et al. used ($df = 419$, $F = 3.6892$, $P=0.0057$). As can be seen clearly, none of the pheromones brought about the dramatic repression seen in this paper, with even an opposite trend seen in the pentacosane treatment group (pairwise test results in Table 62 in the appendices). A commercially available mixture of BP was also used in order to check whether the BP mixture produced in the lab was the cause of the error, however no statistically significant difference between the BP mixtures was obvious. The lack of repression brought about by QMP is not surprising, given that the concentrations to which the flies are being exposed in this experiment are vanishingly small, and that, as can be seen in Figure 5.7, a concentration of even 3.25 Qe of QMP is unable to bring about reproductive repression in this species.

Similarly, the strain used in this paper was the CantonS wild-type strain, which led us to hypothesise that there may be different strain-effects of these pheromones, as it is known that there are differences in effect size and response between different strains of flies on a number of different biological characteristics (such as life span (330) or diapause sensitivity (199)). For this experiment, the methodology used for exposure and ovary measurement was the same manner as has been established prior for the exposure of BP, EBO, and QMP in sections 5.3.2.1 and 5.3.2.2, so as to provide more easily interpretable data relative to that produced in this lab context, as was the concentration of pheromone used.

CantonS flies were exposed to these pheromones using the 2-day fly exposure assay (section 2.4.1.2) presented in Figure 5.10 ($df = 279$, $F = 11.553$, $P<0.001$), where it is clearly shown that neither BP nor pentacosane had suppressive effects on ovary activation (results of individual pairwise tests in Table 63 in the appendices).

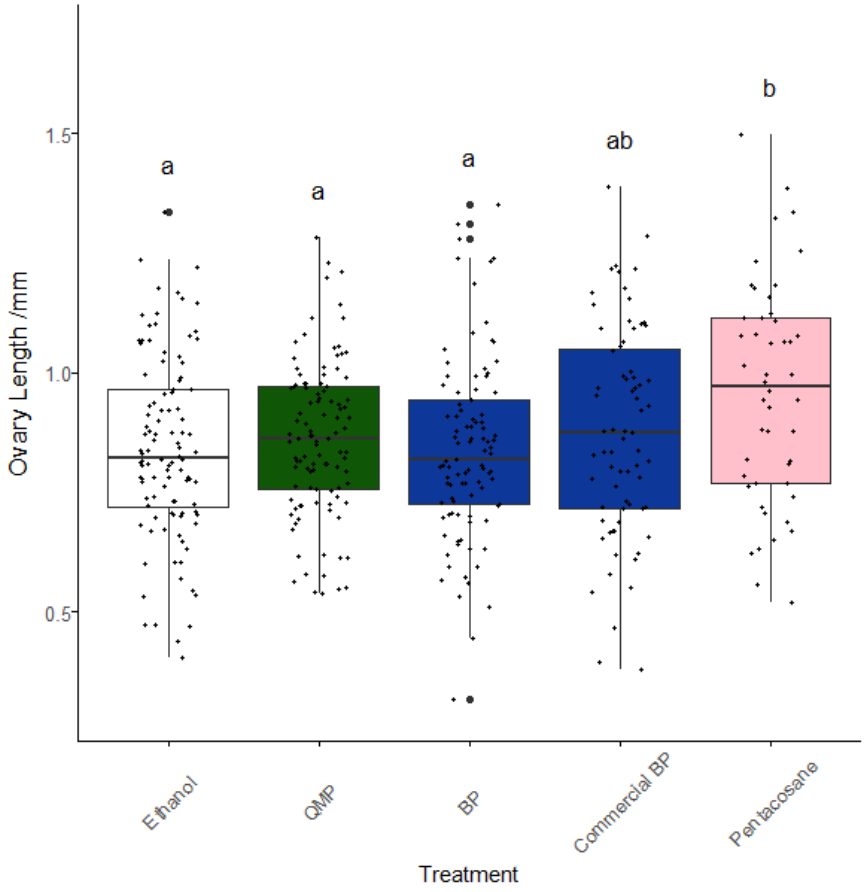


Figure 5.9 – Showing the response of wild-type OregonR virgin female flies to different insect pheromones: QMP (Queen Mandibular Pheromone), BP (Brood Pheromone), Commercial BP (Brood Pheromone sourced from a commercial supplier), and Pentacosane. The application of pheromone was as reported in Velasque et al. 2021 (135). The y-axis shows the ovary length in millimetres and the y axis shows treatment, with Ethanol being the solvent negative control (also containing heptane). Significances are given as letters with P <0.05, calculated via GLM and post-hoc pairwise Tukey tests (n = 424)

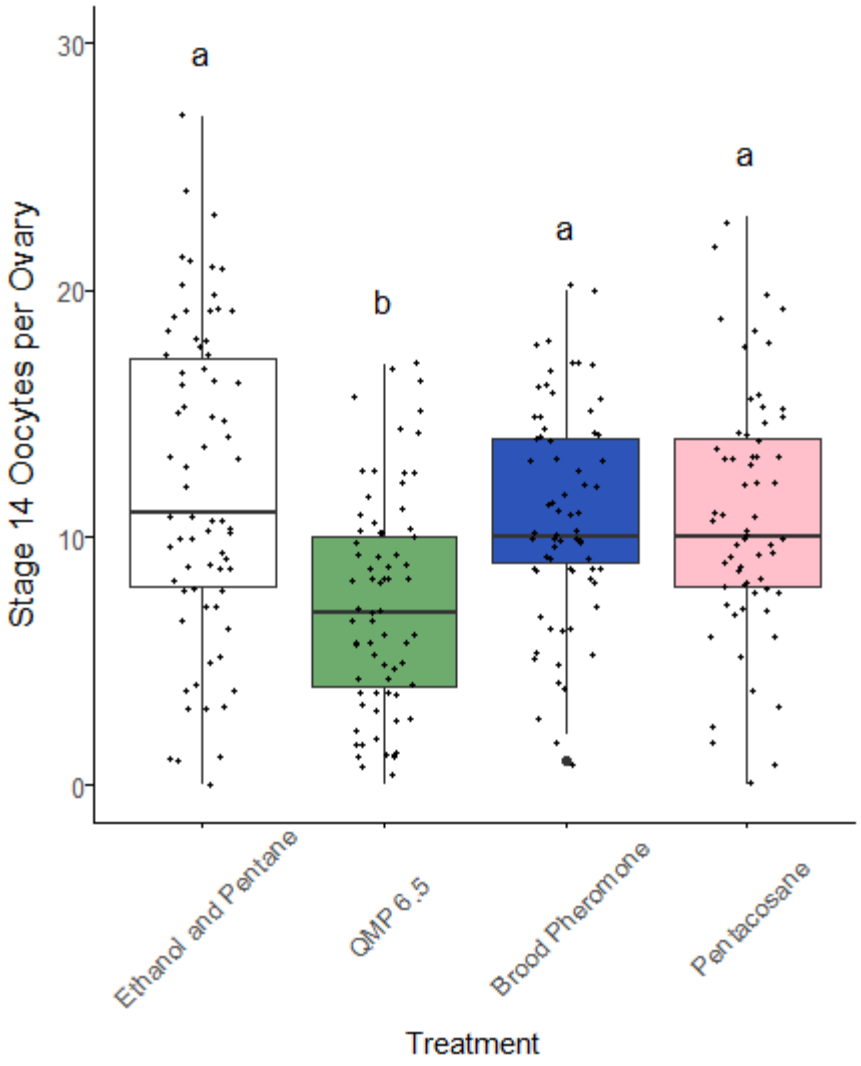


Figure 5.10 – Showing the response of wild-type CantonS flies to three pheromones previously exposed: QMP, BP, and pentacosane. The y-axis shows number of stage 14 vitellogenic oocytes per ovary, and x-axis shows different treatments. 6.5 Qe QMP, 617 Leqs BP, and 232.5 µg of pentacosane (1 Qe). Significances are given as letters P<0.05, calculated via GLM and post-hoc pairwise Tukey tests (n=283).

5.3.2.4 Plant polyphenol quercetin does not inhibit QMP activity in *D. melanogaster*

It has been suggested that the plant polyphenol quercetin has the ability to inhibit QMP-mediated ovary repression in bees (322). Given the robust response to QMP in *Dm*, an attempt to reproduce the inhibitory effect of quercetin on QMP was made in *D. melanogaster* and is shown in Figure 5.11 (df = 483, χ^2=160.65, P<0.001) (pairwise test results are shown in Table 64, in the appendices). As can be seen in this figure, there is no inhibition of ovary repression via QMP by quercetin at any concentration. This shows that quercetin does not act as an inhibitor of QMP-mediated repression of ovaries in this organism.

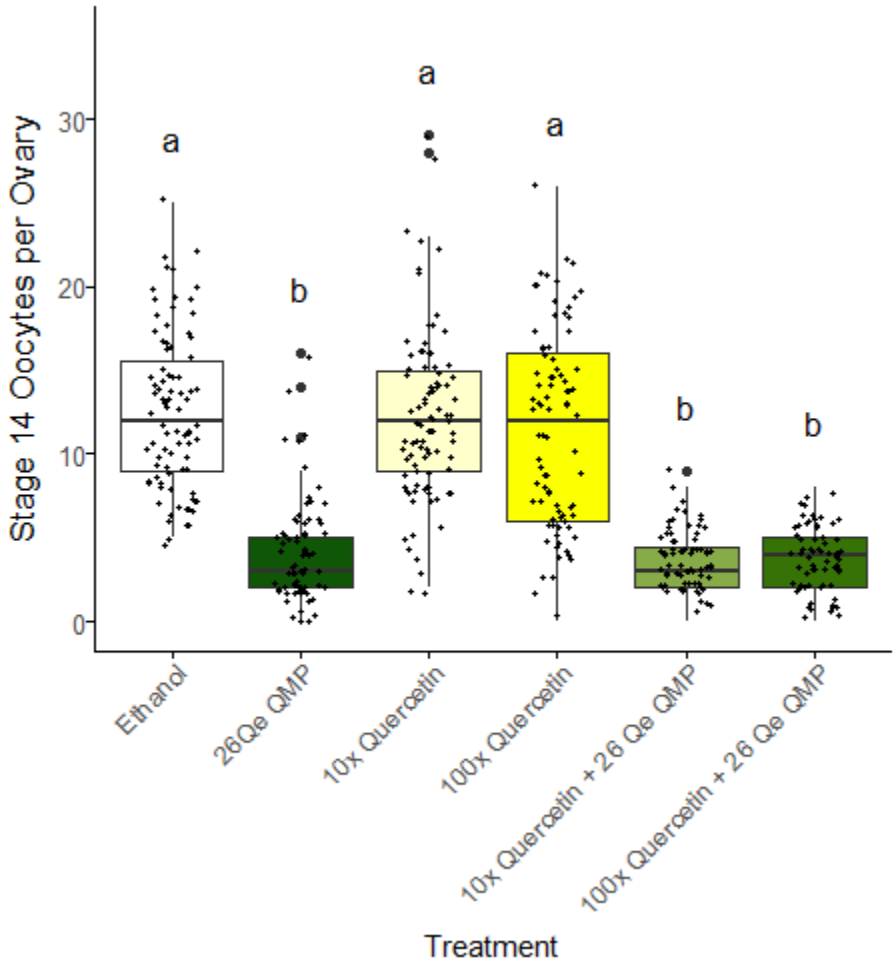


Figure 5.11 – Showing the influence of quercetin on QMP-mediated repression in wild-type OregonR flies. The y-axis shows the number of stage 14 vitellogenic oocytes per ovary, and the x-axis shows the different treatment groups: Ethanol negative control, QMP positive, with two concentrations of Quercetin (100x is 0.01%(w/w) in 30% sucrose, 10x is one tenth the concentration), Quercetin was also applied alongside QMP to investigate antagonistic effects. Significances are shown as letters $P<0.05$, calculated via GLM and post-hoc pairwise Tukey tests.

5.3.3 Effect of quercetin on QMP-mediated reproductive constraint is uncertain

Although quercetin is unable to inhibit the effect of QMP-mediated repression in *D. melanogaster*, this may simply be due to the fact that ovary repression brought about by QMP acts via different mechanisms at some level between *D. melanogaster* and *A. mellifera*.

As a result, this was investigated in an *in cavea* context, in order to validate the findings in *A. mellifera* published by Jie et al. (322).

Due to time constraints only the preliminary work was completed, and is shown in Figure 5.12**.**

In panel **A** of this figure, we see there are no significant differences between any of the samples, and no clear indication of any trend whatsoever (χ^2= 2.3801, df=3, P=0.4974; individual pairwise comparisons in Table 65, in the appendices). Notably all cages exhibit a "suppressed" phenotype very similar to the QMP cages of other experiment (see Figure 5.3), presumably due to toxicity effects of DMSO on the bees. It is also very clear that the samples sizes are simply not large enough to draw any conclusions.

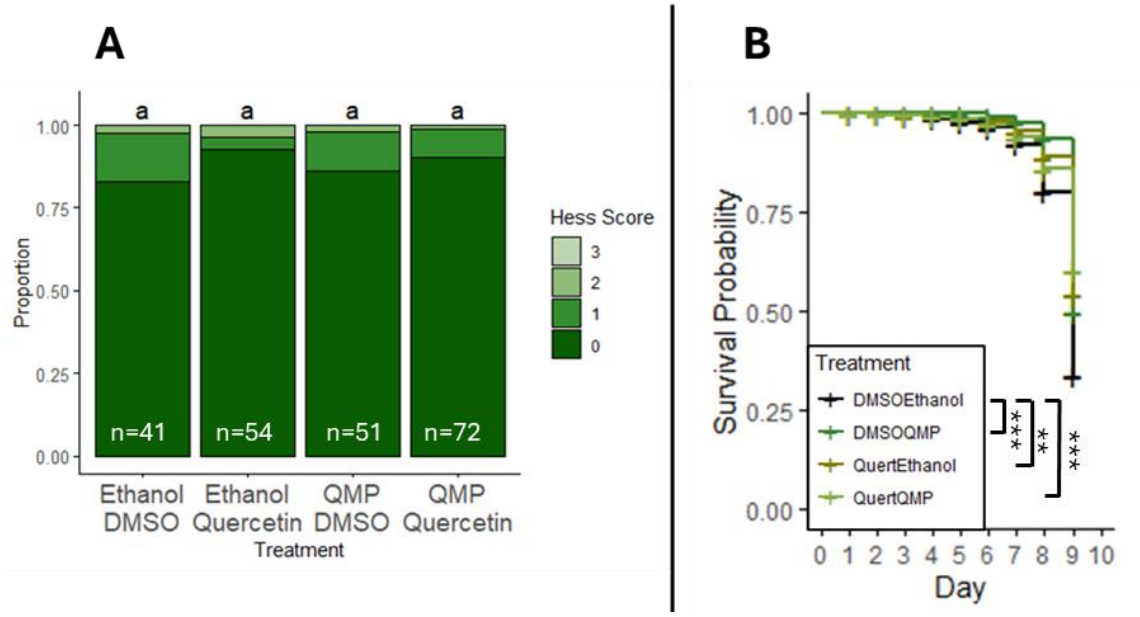


Figure 5.12 – Showing the results from the honeybee quercetin experiment. **A** shows a stacked bar chart where the y-axis shows the proportion of ovaries with Hess score 0-3 (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present), with treatment on the x-axis (Ethanol and DMSO are solvent controls for QMP and Quercetin respectively); the significance is given as letters P<0.05, calculated via CLM and post-hoc Tukey pairwise test. **B** shows a Kaplan-Meier survival curve with survival probability on the y-axis and day of experiment on the x-axis; significance given as **=P<0.01, ***=P<0.001, calculated via CoxME with Tukey post hoc pairwise test.

The mortality curve presented in panel **B** similarly shows similar mortality between the treatment groups, with the exception of the control cage containing no pheromone treatment. It is likely that this is an artifact brought about by the single biological repeat and small sample sizes per cage (χ^2=27.84, df=3, P<0.001; pairwise comparisons in Table 66, in appendices).

Ultimately this preliminary data does not show a clear trend from which we can infer the reproducibility of the original findings. High mortality in cages, as well as low sample size make no obvious conclusions possible.

5.4 Discussion

The role of QMP in the repression of insect reproduction has been discussed at length, both in this thesis and in the general literature. However, the role of other pheromones and interactions is far less studied.

In honeybees, other reproductively repressing pheromones have been identified (158, 160, 324), but neither the cross-species activity of these pheromones, nor their mechanism of action had been investigated. The purpose of the experiments in this chapter was to reproduce the original findings that BP and EBO were able to bring about repression of honeybee worker ovaries, in order to investigate their mechanism of action and compare to the mode of action of QMP.

Additionally, as QMP is able to so broadly bring about repression of reproduction, by testing the mode of action in BP and EBO in fruit flies, we could assess the cross-phylogenetic activity of honeybee pheromones in general against the broadness of QMP.

5.4.1 Brood Pheromone and E-β-ocimene in *D. melanogaster*

Fruit flies are able to have their ovarian activity repressed by the presence of QMP (148, 265, 316). This is thought to possibly be as a result of sensory exploitation: of QMP components having evolved to coopt fundamental signalling pathways within honeybee workers, which are so essential that the control of honeybee worker ovary repression by QMP cannot be easily overcome by worker evolution (128).

In this model, fruit fly sensitivity to QMP is as a result of these shared pathways due to their fundamental importance.

By investigating the effect of BP and EBO in fruit flies therefore, we can see if QMP is uniquely widely acting, and whether BP and EBO are similar to nC25 in their inability to bring about reproductive repression in *D. melanogaster* (134). If BP and EBO had been able to bring about repression of fruit fly ovaries in the manner of QMP exposure, then we can draw broader conclusions about the nature of pheromonal mediated of reproductive constraint in the honeybee as a fundamental rewiring of internal honeybee signalling for the sake of the maintenance of eusociality.

Thus, the findings that BP and EBO are unable to bring about reproductive repression of fruit ovaries, similar to nC25, is consistent with the idea that QMP is unique amongst the honeybee pheromones in the broadness of species in which it is able to act. This gives greater evidence towards the idea that QMP is particularly unusual in the maintenance of the eusocial system of

honeybee social organisation, when compared to other honeybee larval pheromones, and other eusocial queen pheromones, by its uniquely broad phylogenetic effect on reproductive repression. Though these results must also be contextualised within the knowledge that this BP and EBO also failed to bring about repression in honeybees, and it is possible therefore that these pheromone mixtures use *in cavea* were simply not valid examples of honeybee larval pheromones.

These are not the only proofs of this conclusion however, as the differential response seen between the QMP-dose response, and the BP and EBO dose-response demonstrate a different mode of action between the two pheromone mixtures, between *A. mellifera* and *D. melanogaster*. In the QMP dose response we see a very clear linear relationship between QMP concentration and ovary repression. Although the concentrations of QMP are spread over more than an order of magnitude, from 0-26Qe, we do not see the extreme toxicity effects that we see in the BP and EBO samples over similar concentration ranges. In both EBO and BP we see a sharp increase in mortality, with no reduction in ovary activation before high toxicity effects result in loss of ovary activity.

The BP mixture used contains all of the reported components of honeybee BP, but the concentrations may have been an issue, and the ocimene used is the same as that used in the experiments in the literature (160). The application of both of these mixtures over such a large concentration range should have been enough to compensate for the appropriateness of the mixtures.

The fact that QMP does not produce this intense toxicity-induced mortality and effect, but rather has a simple linear concentration response, is indicative that their mode of action is different between the honeybee queen and larval pheromones in this species. It also indicates relatively clearly that QMP is not inducing ovary repression via toxicity effects in this species.

That BP and EBO are able to bring about these toxicity effects in this species indicates that even were the mixtures to be validated in honeybees, we would likely also see significant toxic effects in this species.

5.4.2 BP and EBO in *A. mellifera*

BP and EBO have both previously been shown to induce ovary repression in honeybees, according to Le Conte et al. (160, 324), amongst many other biological functions from other research groups (157, 163, 164, 246, 314, 331). Despite the publication of this phenotype, there have been no publications confirming or refuting this repressive effect on honeybee worker reproduction from other research groups.

5.4.2.1 Brood Pheromone

Due to the lack of reproducibility demonstrated in the literature, the lack of repression of honeybee worker ovaries shown from BP exposure in this chapter is difficult to characterise within the broader literature. It is eminently possible that there is some methodological element of these experiments that has resulted in the lack of BP effect on reproduction, and although many aspects of the methodology were investigated in order to explain the lack of effect of BP in our setup, not all aspects were tested, and various elements were not feasible to test in our setup. The outline of our methodological process is outline in Box 2. Key elements of this pathway that may have significant effect, but which were unfeasible to test in our setup are the genetics of our stock, and the social context in which they're reared, are discussed below.

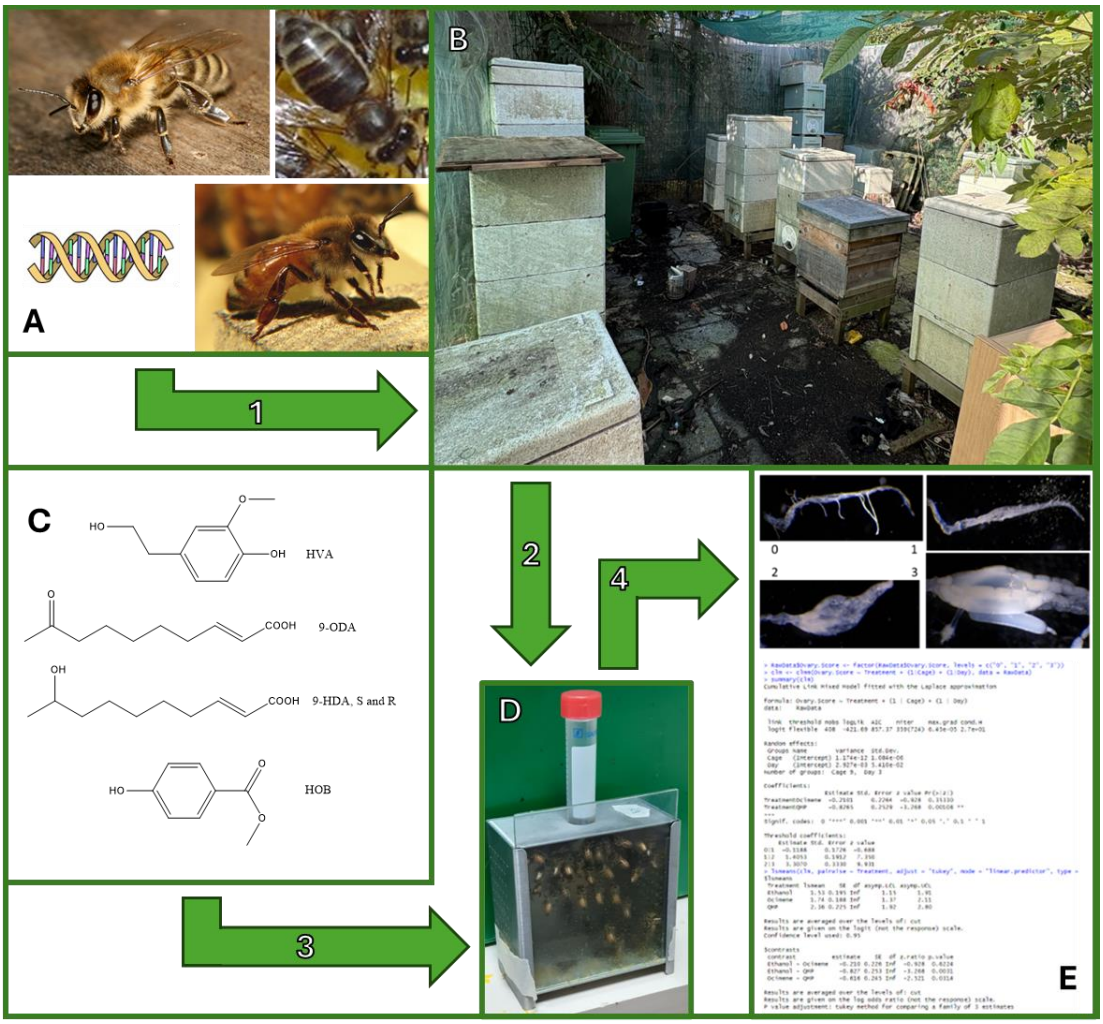
5.4.2.1.1 Strain and genetic effects, and their intersection with pheromone mixture and concentration

Our honeybees have been shown to be a mix of *A. mellifera ligustica*, and *A. mellifera carnica* (the Italian and Carniolan honeybees respectively (332)), though at various points in the three years over which this research was carried out, there have been examples of the *A. mellifera mellifera* subspecies (the British Black; identified by, darker colour, lower brood production, higher propolis utilisation, less honey storage, less sensitive to cold, greater mix of pollen in brood patches). The genetic mixtures of our bees are likely to have changed a great deal over time too, as at any point the number of hives in our apiary was between five and 13, while in a given year, most hives were naturally requeened at least once: as a result of natural supersedence; deliberate introduction of locally-reared queens from beekeepers; swarming; capture of wild swarms and introduction into our stock; or splitting and merging of colonies.

When selecting frames of brood for conducting experiments, although certain colonies were favoured due to their relative strength, almost all hives had brood frames removed at various points, and bees were mixed between these frames for *in cavea* experiments.

The complex genetic background of these hives, and the mixing of bees between hives, allows us to investigate the effect of pheromones as a more aggregated response, limiting the colony-of-origin effects that otherwise obfuscate broader understandings of the fundamental aspects of the mechanism of these pheromones. However, if the BP effect on reproduction is highly variable between even closely related hives, as may very well be the case (307), then it is possible that selecting an appropriate mixture or concentration of BP to demonstrate effect is impossible in our methodological setup. If this were the case, we would expect to see some

degree of ovary repression in the BP-exposed honeybees, but at a reduced level seen in the literature, however this doesn't seem to be the case (Figure 5.3).



Box 2 – Showing the experimental pathway for all BP and EBO experiments, with the potential points of issues labelled with letters, and numbers used to demonstrate experimental flow. **A** shows the genetics of the bees, with the three strains present in our colonies shown going clockwise: *Apis mellifera carnica* (333), *Apis mellifera mellifera* (192), *Apis mellifera ligustica* (334). **B** shows the apiary in which the hives are situated, and from which pupae brood frames are taken to use for experiments. **C** demonstrates the pheromones (here shown as the components for QMP), and the mixtures used to simulate artificial pheromones within a lab context. **D** shows the cage setup and represents the day-to-day experimental procedure through which the experiments are carried out. **E** shows the ovary scoring and statistical analysis, representing the data collection and processing steps.

It is additionally possible that the bees in our apiary are simply insensitive to BP, and a much higher concentration of BP than that used by Mohammedi et al. is necessary to bring about any effect (324).

This was not undertaken in these experiments due to time constraints, however this would be a logical next-step in investigating potential methodological issues with BP sensitivity. It is known that QMP-mediated repression of reproduction does not show a linear dose-response however, particularly that higher concentrations of QMP do not bring about repression, and that QMP-mediated repression demonstrates a U-shaped response (104). So a wide range of concentrations would have to be attempted, in order to avoid potentially over-dosing, or under dosing such that BP has no effect.

5.4.2.1.2 Possible cage effects

It is also possible that the conditions *in cavea* do not represent a natural enough setting for the activity of BP to induce ovary repression. It has been observed that QMP is unable to bring about total repression of honeybee ovaries *in cavea*, in contrast to the almost total repression that exists *in alvo*. This is explainable by the lack of the other more redundant elements of reproductive constraint (47), however it is also possible that this too is caused by the relatively unnatural set up of the cage assay.

The lack of a queen, and her other CHC and pheromone components; the lack of physical brood; and the material out of which the cage is made all provide a more sterile environment in which to investigate the specific pheromone or condition of the experiment. I*n cavea* setups however, also eliminate any holistic, or synergistic effects of the conditions with one another.

For example, it is possible that the lack of brood present to initiate e.g. nursing behaviour, results in the workers developing more quickly into foragers. This would then result in loss of sensitivity to BP, as the honeybee worker has no need to respond to these pheromones in the absence of brood *in alvo*.

It is also possible that the lack of wax present *in cavea*, or the metal and glass construction of the cage results in lack of impregnation of the BP into the environment of the bee, thus limiting its effect (this was theorised as the possible reason for lack of BP activity, and justification for the “in-box” experiments).

A simple, though time consuming, experiment that was planned but not undertaken due to time constraints, is to extract the brood pheromone of the bees in our setup using a similar methodology to that initially used to characterise BP in the literature (335). By identifying the components present in our hives (e.g. by GC-MS), and using this extract to attempt to repress

the reproduction of bees *in cavea*, we could both determine whether the mixture seen in our colonies is consistent with that published in other colonies (160, 307, 328); but also see if larval extract is able to bring about repression of our honeybees *in cavea*.

5.4.2.2 EBO effects

Although the ovary repressive effects of EBO were also unable to be reproduced, only two experiments were undertaken to this intent due to resource and time limitations.

The second experiment, undertaken at the very end of the beekeeping season in September of 2022, demonstrated a statistically significant inhibition of the winter-bee phenotype in the EBO treatment cages. This phenotype, whereby significant metabolic, physiological, and behavioural changes occur in order to facilitate the hibernation of the colony over the winter months, is thought to have been instigated via lack of protein availability at the end of the foraging season (due to lack of flowering plants providing pollen sources). The reversal of this via EBO may be indicative that pollen source starvation may result in a change in behaviour from the queen, and that the lack of brood this brings about is the trigger of the winter phenotype in workers. I.e. that the lack of brood, and thus the lack of EBO, results in the lack of inhibition of the winter phenotype and the transition into winter diapause.

If it is truly possible to prevent the winter phenotype from developing in workers, then it may be possible to elongate experiments into the early autumn via the additional use of EBO *in cavea*. It would not prolong the season overmuch, due to the lack of developing brood, but it would provide opportunities to investigate specific elements of the winter-phenotype during months which would otherwise not contain any available research for honeybee science.

5.4.2.3 Biological relevance of BP and EBO-mediated reproductive constraint in *A. mellifera*

The inability to reproduce the findings of Le Conte et al (158, 315), in the mediation of reproductive constraint via BP and EBO, contrasts very strongly with the ease with which QMP-mediated reproductive constraint is able to be reproduced (47, 96, 104, 111, 123, 132).

Although there are potential methodological obstacles to overcome with the use of BP and EBO *in cavea*, those obstacles seem not to exist for the consistent repression of honeybee worker reproduction via exposure to QMP. The importance of QMP as a method by which eusociality is maintained in this species cannot be overstated, however it is notable that the application of BP in the two papers demonstrating the effect of the mixture on reproductive repression, shows a greater effect size on ovary repression than QMP.

The fact that this magnitude of effect size could not be reproduced *in cavea*, using the same mixtures and similar methodological approaches, perhaps reflects that the biological relevancy of this phenotype may be overstated in the original papers.

If it were truly an important effect, then it could be argued that it should be able to cut through the methodological issues presented in this chapter, in order to demonstrate an effect of any sort. The lack of this effect simply implies that BP and EBO, while important for other biological effects *in alvo*, particularly in role selection of honeybee workers, are not important elements for maintaining reproductive constraint in this species.

5.5 Conclusions

The biological relevancy of BP and EBO as mediators for reproductive constraint in *A. mellifera* have been brought into question. The lack of reproducibility of the published data, as well as the lack of activity in *D. melanogaster*, imply that the importance of these compounds within the eusocial structure of honeybees is less than previously suggested.

There are additionally, no significant mortality effects of these compounds in honeybees, but there are in fruit flies in response to these compounds.

The paper in review on BioRXiv by Velasque et al. (135, 321) cannot be reproduced in our lab context.

Additionally, quercetin does not seem to be able to inhibit QMP activity in fruit flies, but methodological difficulties, and time constraints, prevented adequate attempts to investigate this *in cavea* in honeybees.

5.6 Future Work

5.6.1 Brood Pheromone and E-β-ocimene

In order to understand why there is a difference between the published data and the *in cavea* experiments conducted here with regards to the repressive effect of BP and EBO, there are several possible avenues for investigation.

First, by extracting and characterising the larval pheromones produced by our own bees (by age matching larvae and washing them in hexane and ethanol to dissolve compounds, the extracts can be applied directly to our bees, but also characterised using GC-MS), we can perhaps eliminate any concentration or mixture influences on reproductive repression.

In a similar vein, different concentrations of the BP mix published by Le Conte et al. would be a simple experiment to eliminate this variable. Additional changes to methodology are also possible, such as introducing comb into the cages, or material of cages, in order to introduce possible synergistic effects which potentiate the workers to the activity of BP and EBO as reproductively constraining compounds.

It is also possible to investigate the effect of strain by sourcing bees from the original research institution which published the original findings. By conducting the experiments on these bees, we would be able to eliminate the possible strain effects postulated.

A second direction of enquiry would be to attempt to use the alternative effects reported in the literature in response to BP and EBO, in order to show that our workers are able to respond to these compounds. By, for example, attempting to measure changes in sucrose response threshold (331), we can use these other effects to validate the mixture itself in order to narrow down possible extraneous effects for why these compound mixtures did not produce a reproductively repressive effects in our *in cavea* setup.

Similarly, looking at gene expression via RNAseq or RT-qPCR, looking at possible gene expression changes associated with those reported effects of BP and EBO activity would achieve similar results (such as genes in the brain of the honeybee associated with forager behaviour, like *dop2*), and would help to characterise the mechanism by which BP and EBO are able to bring about changes in the physiology and behaviour of adult workers.

Chapter 6 – What is the mechanism of action of QMP in *D. melanogaster*

6.1 Introduction

The honeybee pheromone QMP, produced in the mandibular glands of honeybee queens, has been shown to elicit similar reproductive repression in *D. melanogaster* as in its own species (148, 183, 316).

The phenotype observed only occurs at much higher concentrations of QMP than *A. mellifera* workers: *in cavea* honeybee workers are suppressed in their reproduction at 0.1Qe of QMP per day, however a concentration of 3.25Qe is insufficient to bring about statistically significant repression of fruit fly ovaries (Figure 5.7).

The wide-acting nature of QMP activity in species only distantly related to honeybees, raises questions about the nature of the mechanism of action of this pheromone mixture in the honeybee and in the fruit fly. Possibly some fundamental mechanism of insect, or animal, biology has been coopted by QMP in honeybees via the sensory exploitation hypothesis, and that this results in co-option of similar mechanisms in other, only distantly related species. For example, it is known that QMP affects Notch signalling in the honeybee ovary, which is a highly conserved signalling pathway across the insects, and so may be a key element in the broad activity of QMP (132, 138). Alternatively, fruit flies are known to behave in a starvation-like manner when exposed to QMP (183), and honeybees have been shown to eat more carbohydrates in exposure to QMP, so possibly carbohydrate metabolism is involved (Figure 3.4).

6.1.1 *D. melanogaster* as an investigative model

The fruit fly represents a useful model organism for molecular, developmental, and physiological investigation. They are also easy and quick to rear large numbers of genetically identical individuals (336). A large number of genetic techniques have also been developed over the last 100 years to investigate various elements of the biology of these insects, such as the UAS-Gal4 system (320), RNAi (337), or the development of chromosome balancers (338); techniques which have yet to be developed, or are much more difficult to implement, in the other species investigated in this thesis (339). There are also far larger bodies of research in the fruit fly that allow us to better contextualise our investigations within the broader understanding of the organism (for example within the molecular underpinnings of diapause (199)).

If the mechanism of action of QMP is the same in the fruit fly as it is in the honeybee, or uses related mechanisms between the two species, then we gain the ability to investigate the action of QMP in the honeybee using the far deeper toolkit of techniques that are present in the fruit fly.

6.1.1.1 The UAS-Gal4 system

One of the key techniques that allows such great control of fruit fly biology for investigative research is the UAS-Gal4 system (340), and is laid out simply in Figure 6.1.

In this system, a Gal4 protein binds to an upstream activating sequence (UAS) to initiate the transcription of the gene of interest. By using transposon elements to introduce a gene of interest (GOI) downstream of this UAS element, we produce a fly line which has an inducible GOI in the presence of Gal4. Recently, a refinement of this technique uses attB/P elements to achieve this more consistently and with less variance in result (341).

We can then produce a second fly line, which produces Gal4 only in specific tissues. The Gal-4 protein can be linked to another gene which is selectively expressed in specific tissues, or at specific points in development of the fly. By inserting the Gal4 gene downstream of this other gene's promoter, we can piggyback the expression of Gal4 with this tissue specificity (342).

Alternatively, Gal4 can be expressed temporally by including it downstream of a temperature-sensitive promoter, such as a heatshock promoter, and so can be induced at any time in development called for by the experiment (343). Gal4 expression by itself does nothing in the fly genome (it is repurposed from yeast genetics), and so has no deleterious effects on fly health (320).

By crossing the Gal4 and the UAS lines together, we are able to get selective expression of a GOI in a chosen tissue. Additionally, by choosing GOIs which are already present in a tissue, we can overexpress that gene in that tissue, which allows for investigation of overexpression of given signalling pathways or metabolic processes.

Within a given metabolic, or signalling pathway, there are often inhibitory and repressive elements. By targeting these specific proteins to overexpress, we can either increase the activation of the pathway, or decrease its activation. For example, in the insulin signalling pathway scheme shown in Figure 6.2, we can overexpress Akt to inhibit Foxo signalling and to activate Tor signalling. We could also overexpress Foxo to inhibit the effects of insulin signalling in a tissue, without affecting Tor signalling directly.

By selecting genes which have activating functions we can therefore increase signalling of a given pathway, or by selecting genes with inhibitory functions we can repress signalling of a given pathway.

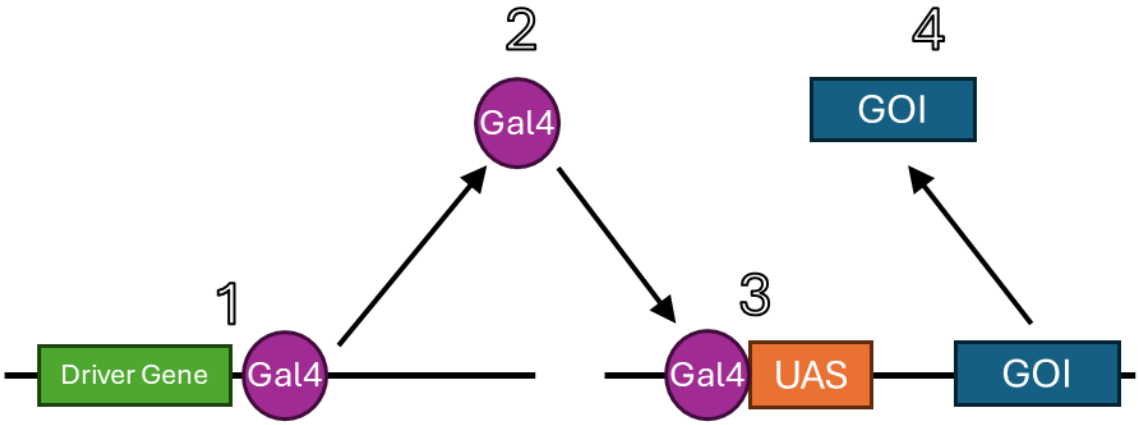


Figure 6.1 – Showing a rough outline of the UAS-Gal4 system in fruit flies. A driver gene, which has native expression in a given set of tissues or developmental periods in the fruit fly, can be utilised to co-express the Gal4 protein shown in **1**. The free Gal4 (**2**) is then able to bind to the UAS region of my gene of interest (**3**) and cause the expression of that gene of interest in any tissue in which the driver gene is also expressed (**4**). (340)

6.1.2 QMP activity in the fruit fly

QMP activity in the fruit fly has been studied previously. Galang et al. identified certain antennal odorant receptors (Or-56a, Or-49b and Or-98a) are activated in the presence of QMP (316), however Lovegrove et al. found that antennae and maxillary palps can be removed from fruit flies and this does not inhibit QMP activity in this organism (111), suggesting that antennal odorant receptors are not the main mechanism by which QMP is detected in this organism (this does not exclude the gustatory receptors as the mechanism by which QMP is detected (344)).

Lovegrove et al. also showed that fruit flies respond to QMP exposure in a manner similar to starvation, and with equivalent changes in insulin signalling (183). This possibly represents an example of sensory exploitation in the fruit fly, whereby starvation mechanisms have been coopted within the honeybee to bring about repression of preproduction by QMP, and that these metabolic and signalling pathways are so conserved that it results in QMP activity in the fruit fly. This may also be a good example of diapause mechanisms specifically having been coopted.

D. melanogaster are also known to display a great deal of plasticity in their reproductive activity (199). They are known to be able to enter a reproductive dormancy state known as diapause (199). As has been shown in Chapter 3, honeybees do not possess a plastic adult QMP-mediated response to ovary repression once their ovaries are active, although the same experiments have not been undertaken in fruit flies. Whether the general plastic response seen in fruit flies exhibits in a more flexible manner to honeybees may hint at differences or similarities between the biological response of these two species to QMP.

6.1.3 Diapause in fruit flies

Diapause is the mechanism by which insects are able to bring about changes to their physiology, metabolism, and anatomy that allow them to maximise their reproductive fitness throughout the course of their lives in response to environmental stressors (195-197). By waiting for a period in which reproduction results in maximised number of offspring, and in that waiting shutting off reproduction and switching metabolic processes over to a mode which maximises longevity (345), they can increase their reproductive output over the course of their lives.

Diapause in *D. melanogaster* can be investigated in a lab setting via the use of cold and shortened day light cycle (198), and indeed this technique has been used to demonstrate that insulin signalling changes in response to diapause (199). This allows us to make comparisons between the QMP-induced reproductive repression we see in the fruit fly and cold-induced diapause in order to investigate the differences in the responses (if any exist). By inducing diapause via cold, and separately inducing reproductive repression by QMP, we can compare and contrast the differences in phenotype between the two. We can also see if disruptions to diapause, e.g. through mutants which lack, or have disrupted, diapause, also disrupt the ability of QMP to bring about reproductive repression.

Additionally, we can interfere with certain internal signalling pathways using the UAS-Gal4 system to bring about overexpression of elements of those pathways in different tissues, to investigate whether specific tissues are involved in diapause and/or QMP-mediated reproductive repression.

6.1.4 Insulin signalling

Given the role of insulin signalling in the QMP-mediated reproductive repression of the fruit fly, and in diapause in this organism, this pathway seems a prime candidate to study for its role in both cold diapause and QMP-mediated reproductive repression. A highly simplified version of the insulin signalling pathway is shown in Figure 6.2.

Various elements of this pathway are promising elements to overexpress in order to investigate if there are any differences in the activity between cold and QMP, e.g. Chico, which acts as an early element of insulin signalling after the receptor activates. By overexpressing this protein, we should be able to upregulate the effect of insulin signalling in a given tissue.

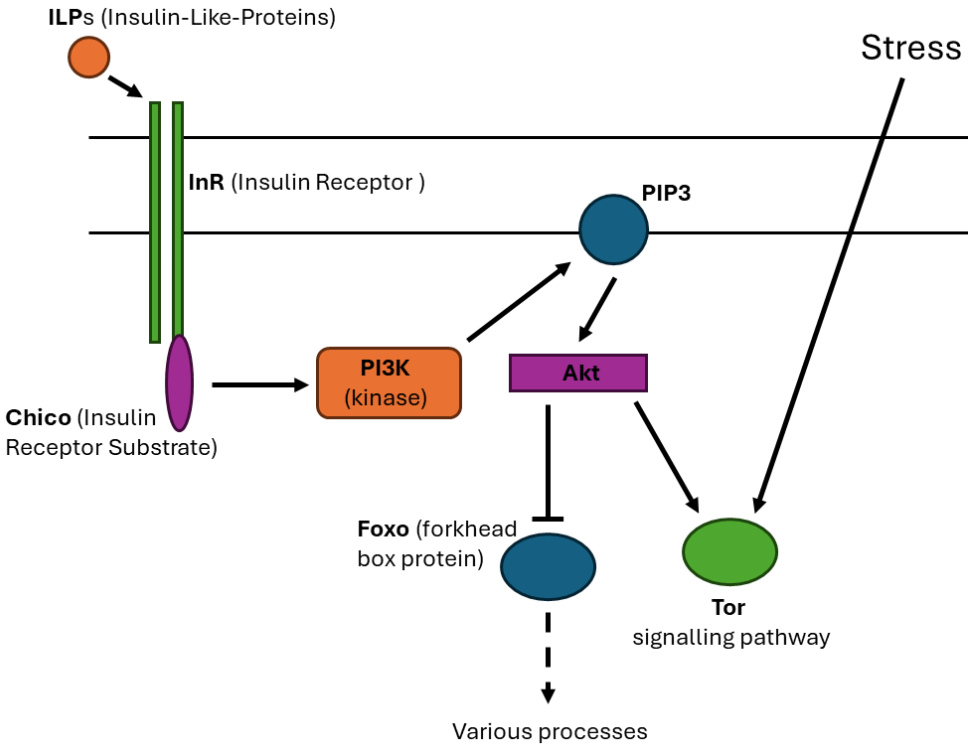


Figure 6.2 – Showing a highly simplified overview of insulin signalling in the fruit fly *D. melanogaster*. Insulin like proteins (ILPs) bind to the Insulin receptor, a tyrosine kinase receptor which then autophosphorylates, allowing the activation of substrate proteins such as Chico. Chico then continues on to activate PI3kinase, which allows the activation of PIP3 in the cell membrane, which acts as an activator of Akt. Akt is able then to mediate a number of different downstream elements such as the inhibition of Foxo and the activation of the TOR signalling pathway. TOR is also activated via other mechanisms, such as somatic stress in the fly.(346)

6.1.5 Research Questions

The research herein will attempt to answer the question, how is QMP-mediated reproductive repression of *D. melanogaster* working?:

- Is the lack of plasticity seen in honeybee ovary repression with exposure to QMP similar to that seen in fruit flies?
- Is QMP-mediated repressing of ovaries in fruit flies working via diapause mechanisms?:
    - Can we demonstrate that cold-induced diapause and QMP-induced repressive reproduction correlate in their effect across strains which have differential cold-sensitivity?
    - Can we demonstrate that diapause-deficient mutant lines are also deficient in QMP-mediated reproductive repression?
    - What role is there in diapause and QMP-mediated repression for insulin signalling, and in which tissues are these mechanisms bringing about reproductive repression?

6.2 Methods

6.2.1 Fly lines

The fly lines used throughout this chapter, and their sources, are detailed in Table 14. The CantonS line was a kind donation from Elwyn Isaac, the Dahomey line from Amanda Bretman, and the fatbody-Gal4 line from Laurin McDowall, at the University of Leeds.

Table 14 – Detailing the sources for all fly strains used in Chapter 6

Strain	Source
UAS-Foxo	Bloomington: 42221
UAS-Chico	Bloomington: 93138
UAS-ILP2	Bloomington: 80936
UAS-InR	Bloomington: 8263
Act/Cyo-Gal4	Bloomington: 4414
sev-Gal4	Bloomington: 5793
MTD-Gal4	Bloomington: 31777
fat-Gal4	Laurin McDowall
Cpo1	Bloomington: 39665
Tim01	Bloomington: 80930
OregonR	Bloomington: 25211
CantonS	Isaac Lab
Dahomey	Bretman Lab
W1118	Vienna: 60000

6.2.2 Investigating plasticity of *D. melanogaster* QMP-response

Virgin female flies were collected and aged for five days. 40 flies were dissected and ovaries fixed as according to sections 2.5.2 and 2.6.2, to validate phenotype ovary activity in the flies. The remaining flies were moved to pheromone assay vials and treated for two days with either 26Qe or ethanol solvent control (as detailed in 2.4.1.2 and 5.2.3) then ovaries were dissected and fixed according to sections 2.5.2 and 2.6.2.1. Ovaries were then analysed according to 2.6.2.2.

6.2.3 Diapause assay for investigating the wild-types, mutant, and parental lines

QMP-diapause was assayed via the 2-day pheromone assay laid out in 2.4.1.2, using 6.5Qe of QMP for each assay tube. This concentration was used as it is the smallest concentration able to bring about significant ovary repression in OregonR (Figure 5.7), thus allows us to see positive and negative attenuation of effect size.

Cold diapause was conducted following the methodology of (199). Virgin females were collected, aged for 24 h and sorted into cold conditions or warm conditions. The warm condition flies were placed on 7 ml of cornmeal media in tubes and kept at 25°C for seven days on a 12:12 Light:Dark cycle. Cold condition flies were placed on the same media and kept at 11°C on a 10:14 Light:Dark cycle, for three weeks total.

For the wild-type experiments cold fly vials were flipped every 7 days onto fresh media (due to concerns about media shrinkage and mortality effects), but this step was omitted for the mutant and parental line experiments where the flies were kept on the same media for all 21 days as the media shrinkage and mortality issues were not observed.

At the end of each assay the flies were dissected, ovaries extracted, fixed and preserved in glycerol, detailed in section 2.6.2.1. The ovary activity was measured by counting the number of stage 14 vitellogenic oocytes per ovary, detailed in 2.6.2.2.

6.2.4 Investigating effect of Insulin signalling in diapause and QMP-mediated reproductive repression

Virgin females of the UAS lines detailed in Table 14, were collected and placed with Gal4 males in a ratio of 5:4 respectively on room-temperature cornmeal media. The flies were flipped onto fresh room-temperature cornmeal media every two days until all females had died. The F_1 offspring was then taken as virgins for use in diapause assays.

The UAS-line was always female, and the Gal-4 line always male. Males were not collected as virgins.

6.2.5 Statistics

All statistics were carried out in R studio (V2024.04.2+764). The graphs were plotted in ggplot2, and finished as necessary in Microsoft PowerPoint. Analysis was undertaken in two parts: one for the raw data to show the absolute ovary activity of the fly strain being used, and one for investigating the effect size to demonstrate the relative ovary activity between the experimental controls and effects.

6.2.5.1 Statistical significance of raw count data

The distribution of the data was first identified using the distrplus package. A Cullen and Fray graph was used to identify possible distribution types, before the data was fitted to different distributions and plotted with analytical graphs. A theoretical density plot, Q-Q plot, CDF plot, and P-P plot were used to identify which distribution was likely to have the best fit.

The distribution was then fitted to a glm. If the distribution was gaussian, this was fit in the base R glm function with covariates identified as biological repeat, day of experiment, and whether the glycerol had worked as intended (named quality). The glm was then plotted via diagnostic plots to check its fitting, then an ANOVA run using an F-test to check for the predictor effect of the covariate relative to null model. The non-statistically significant covariate with the largest P-value was then converted to a random effect variable in the glm function, then the process repeated through to the ANOVA until all covariates were able to statistically significantly function as predictors, or had been converted to random effect variables.

The emmeans package was then used to do pairwise comparisons of the appropriate data, and if multiple test comparisons were used, then a Tukey adjust was applied, otherwise no adjustment was applied.

If the distribution was negative binomial, the glm.nb function from the MASS package was used instead. The process was carried out as above with some differences. This function does not incorporate a negative binomial distribution into a mixed effects model, and so when covariates were eliminated they were not included as random effect variables. The ANOVA used to check the strength of the predictor effect versus a null model used a chi squared test rather than an F test.

6.2.5.2 Cohen's D effect size measurements

The effect size was measured using a Cohen's D function using base R. In order to plot the Cohen's D values of the two diapause types relative to one another, the data was displayed on the same ggplot2 graph, where the smaller QMP-effect size was stretched based on the OregonR values: the two effect sizes for OregonR were normalised to one another, and the rest of the data stretched to fit the same graph. This was done in order to more easily demonstrate whether there was a correlation of the two diapause types. The absolute Cohen's D value for the QMP-exposed flies was lower due to the concentration of QMP used, and as QMP is shown to be dose-dependent (see Figure 5.7, and (265)), higher concentrations of QMP would have brought about a higher effect size of QMP-induced reproductive repression, in a linear fashion.

6.3 Results

The reproductive diapause seen in *D. melanogaster* fruit flies can be triggered by a variety of different environmental and biological conditions (196). It is also known that fruit flies are highly plastic in their response to these triggers (198, 259, 265), though it is not yet known if QMP is able to bring about repression of active ovaries in this species.

The role of insulin signalling and starvation effects during QMP exposure in *D. melanogaster* fruit flies (183), as well as observations regarding the sensitivity of different wild-type fly lines to cold-induced diapause (199), highlights a potential method for investigating whether the reproductive repression induced by QMP is the same form of repression as the diapause induced by cold conditions.

6.3.1 *D. melanogaster* demonstrate a highly plastic response to QMP-mediated reproductive repression

It has been demonstrated in Chapter 3, that honeybees are unable to have their ovaries repressed via QMP once they have been allowed to activate.

In order to investigate this virgin female fruit flies were allowed to activate their ovaries and then exposed to QMP to repress them.

As can be seen in Figure 6.3, after being allowed to activate for five days, QMP is able to bring about significant repression of ovary activation in fruit flies (GLM: $F=165.66$, $df=366$, $P<0.001$; individual pairwise comparisons in Table 67 in appendices). The ovaries can also be seen to have activated by day five, and also that in the absence of QMP the ovaries continue to activate.

This clearly demonstrates that *D. melanogaster* fruit flies possess a highly plastic response to QMP in line with the hypothesis that QMP acts via diapause mechanisms, and is consistent with work produced by Lovegrove (265), showing the ability of flies to recover from QMP-induced repression of reproduction.

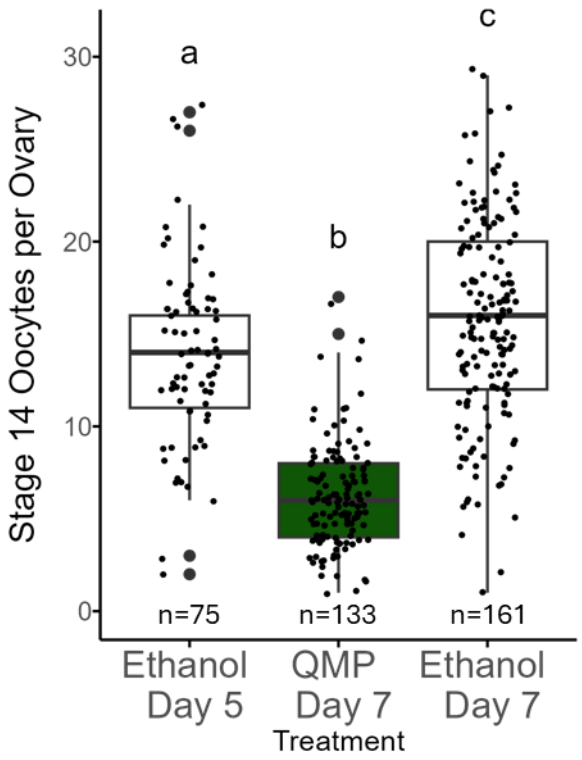


Figure 6.3 – Showing a boxplot of ovary activation in *D. melanogaster* fruit flies which have been allowed to activate their ovaries before being exposed to QMP. The x-axis shows day of dissection after eclosure, with treatment of flies either exposed to 26Qe of QMP or ethanol solvent control. The y-axis shows the number of stage 14 vitellogenic oocyte per ovary. All flies were not exposed to treatment until day 5, after which a subset were dissected to check for ovary activation, and the others moved into vials for exposure. Significance given as letters ($P<0.05$). QMP treatment is shown in green, while control is shown colourless. Fruit flies can have their ovaries repressed via QMP after they have activated.

6.3.2 Wild-Type *D. melanogaster* show a correlation between cold sensitivity and QMP-sensitivity in their diapause response

It has been shown that different wild-type flies exhibit different sensitivities to cold-induced diapause (199). These different sensitivities to diapause in general, can therefore be used to investigate whether these lines also exhibit differential sensitivity to QMP-mediated reproductive repression, and if so, provides us with evidence of diapause being the mechanism by which QMP is able to bring about reproductive repression in this species.

By inducing diapause in different wild-type flies via exposure to cold, in the manner of the aforementioned paper (199), and by exposing these same fly lines to QMP and measuring the difference in effect size of this ovary repression under both conditions, we can investigate

whether the sensitivity to cold and the sensitivity to QMP correlates. This would imply that these two systems utilise the same diapause system to bring about ovary repression.

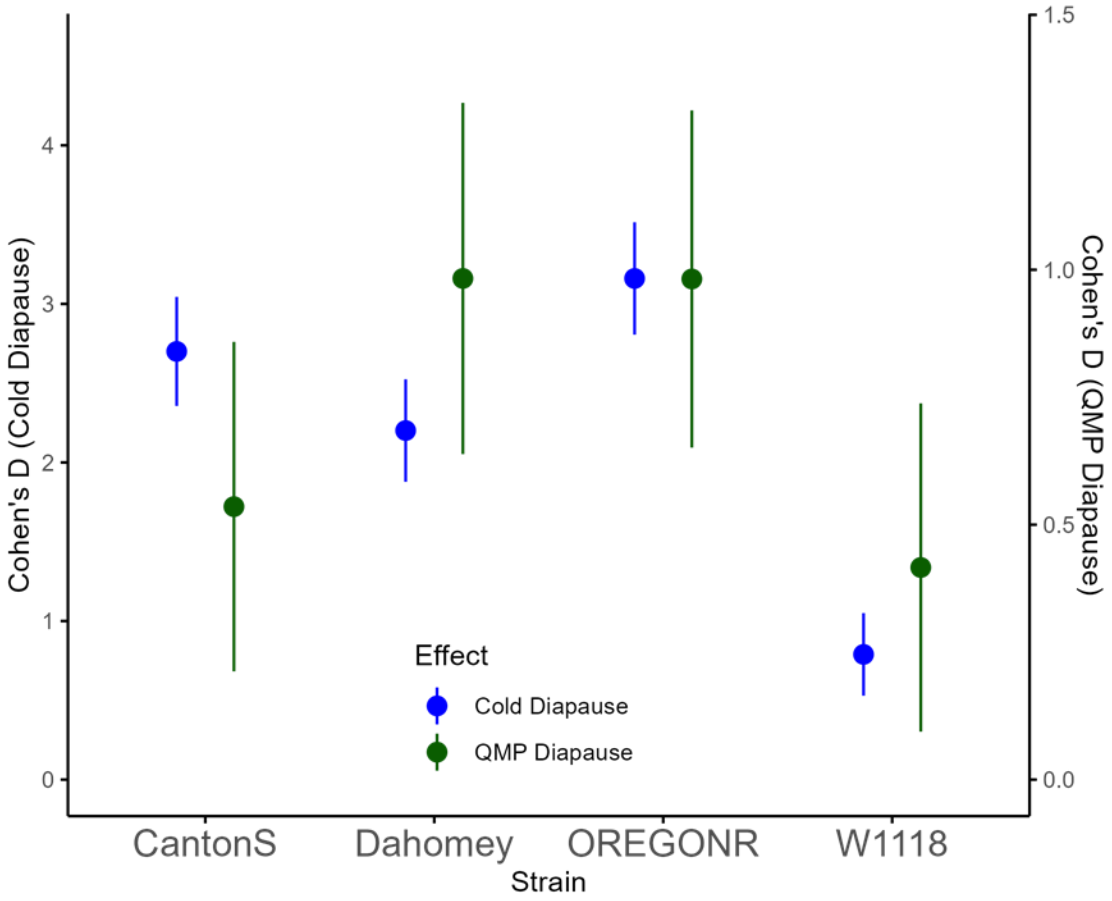


Figure 6.4 – Showing the different effect sizes of cold and QMP induced diapause for different wild-type strains of *D. melanogaster*. The x-axis shows four different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively. There is a correlation between sensitivity to QMP diapause and sensitivity to cold diapause.

As can be seen in Figure 6.4, there is a correlation between these two phenotypes. This is particularly true of the OregonR and the W1118 lines. This effect size correlation is independent of the absolute magnitude of ovary activation, i.e. that the strains with larger ovaries generally do not simply just have larger effect size (Figure 6.5). In this raw data, we can

see that e.g. Dahomey exhibits the same degree of ovary activity in 25°C conditions as CantonS, but has differing sensitivity to cold diapause. The ethanol results for CantonS also contain a high degree of variance, and so Cohen's D is less able to capture the effect size in the manner that occurs for e.g. OregonR., which exhibits a much lower variance. N.B. the two y-axes displayed on this graph are of different magnitudes (see section 6.2.5.2 for more details).

These data suggest that there is a positive correlation between the sensitivity of these two diapause types. Notably however it does not show a clear linear relationship between these two phenotypes.

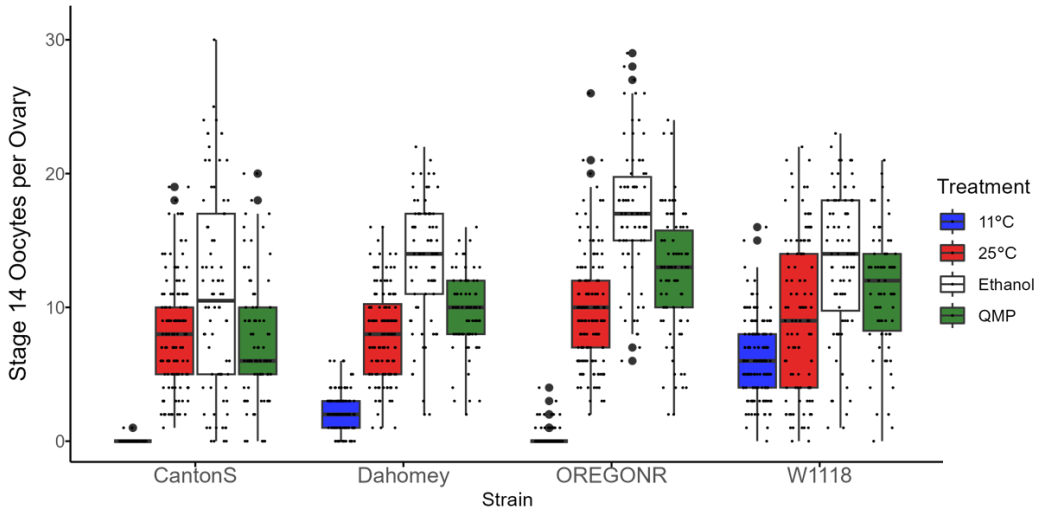


Figure 6.5 – Showing boxplots of the ovary activation of different strains of wild-type *D. melanogaster* to different diapause conditions. The x-axis shows different wild-type strain of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.3.3 Diapause-affected mutants *Cpo1* and *Tim01* also show a correlation between the effect size of cold and QMP-mediated diapause

To investigate this further, Flybase was searched for diapause-affected mutants. From this, two mutant fly lines were discovered with disrupted diapause activity: the *couch potato* line *Cpo1* (347), and the *timeless* line *Tim01* (348).

If these mutants possess disrupted diapause with regards to cold, and also possess disrupted QMP-induced reproductive repression, then this would be further evidence that QMP-induced reproductive repression is mediated via the same mechanism as cold-induced diapause.

As can be seen in Figure 6.6, these two mutants' diapause effect size is reduced relative to the OregonR wild-type, as is their sensitivity to QMP. The raw data in Figure 6.7 shows that their response is characteristic of the response seen before with wild-type strains in Figure 6.5, with no obvious irregularities.

The presence of the same correlation between the two mutants, both of which possess limited diapause response further strengthens the original hypothesis that cold and QMP diapause operate via the same mechanism.

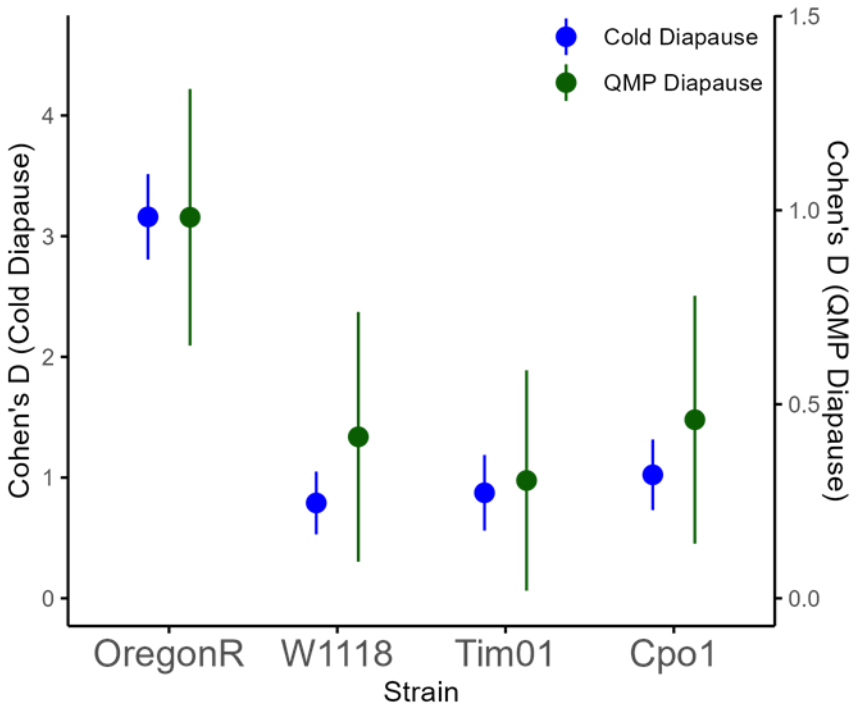


Figure 6.6 – Showing the different effect sizes of cold and QMP induced diapause for different mutant strains of *D. melanogaster*, as well as two wild-types strains. The x-axis shows four different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively. In both strains with mutations affecting diapause, the correlation between cold and QMP diapause is maintained.

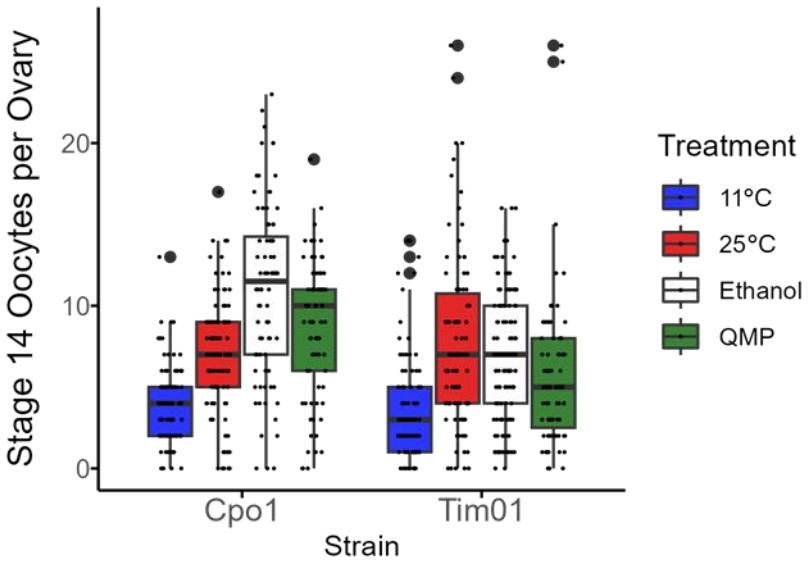


Figure 6.7 – Showing boxplots of the ovary activation of different strains of mutant *D. melanogaster* to different diapause conditions. The x-axis shows different mutant strain of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.3.4 Insulin signalling

Insulin signalling has been shown to be involved in the QMP-mediated repression of *D. melanogaster* ovaries (183), as well as involved in diapause in this species (199). As a result, this was chosen as the mechanism through which the mechanisms of cold and QMP-mediated repression would be investigated.

By using the UAS-Gal4 expression system, we were able to overexpress various elements of the insulin signalling pathway in different tissues, in an attempt to disrupt the correlative effect of cold and QMP-mediated repression.

6.3.4.1 UAS-Gal4 parental line backgrounds have significantly disruptive effects on cold and QMP-mediated reproductive repression

First, as has been shown in section 6.3.1, different wild-type strains have different sensitivities to both types of diapause. The UAS and Gal-4 lines used in these sections are utilising different genetic backgrounds, and so may have different native sensitivities.

The determinant of these sensitivities, may be related to various mutations on these backgrounds, and the ideal controls to compare the efficacy of the crosses on the effect size of cold or QMP diapause would be a cross of the backgrounds of the UAS and Gal4 strains, or of backcrosses of these lines.

However, these backgrounds were not available, and there were time constraints which prevented backcrossing, and so alternatives were used for each strain.

Where possible, the Gal-4 driver *Sevenless* was used as a control for the driver line. This line drives expression in the eye of the fly (349)(as well as latently in other tissues, such as the male germline (350)), and so is often used as a control in UAS-Gal4 experiments investigating other tissues. Otherwise, where the *Sevenless* line did not work (possibly due to off-target effects), the parental lines themselves were used as controls.

The base effect size on diapause and raw activity can be seen in Figure 6.8 and Figure 6.9 respectively.

Unusually, the Act/CyO-Gal4 driver, which drives wherever actin is expressed (as a proxy for systemic somatic expression), shows an inverse response to cold diapause, where the line has more ovary activity under cold conditions (It does not show this in response to QMP, however the pheromone has no effect in this line). Additionally: the fatbody-Gal4 line (Bloomington:2167), which drives expression in the fatbody tissue; MTD-Gal4 (Bloomington:31777) which drives expression in the ovaries; UAS-Foxo (Bloomington:42221), which expresses the Foxo gene; UAS-InR(Bloomington:8263), which expresses the insulin receptor; and UAS-Chico (Bloomington:93138), which expresses the Chico gene; all lack a response to cold diapause, such that they exhibit no reduction in the number of stage 14 vitellogenic oocytes.

Only UAS-Foxo and UAS-InR also lack QMP sensitivity, with all other parental lines not having lost this phenotype.

Of all the parental lines, only UAS-Foxo and UAS-ILP2 possess a correlation between the two phenotypes (though UAS-Foxo shows zero diapause overall). Suggesting that the methods used to generate these line significantly affect the ordinary biology of the organisms.

In the UAS lines we may expect to see some disruption of natural effects, due to the possible “leakiness” of these lines (e.g. (351)). I.e. that these lines can produce a small increase in expression of their target genes unpredictably. Though it is surprising the extent to which the correlative effect has been disrupted.

The reversal of the Act/CyO line is also surprising, and perhaps shows that this line is not as appropriate for measuring cold and QMP diapause effects.

The large variation in effect size of these parental lines, particularly with the well-established cold-diapause response demonstrates the importance with which these lines should be considered when contextualising the phenotypes of the crosses.

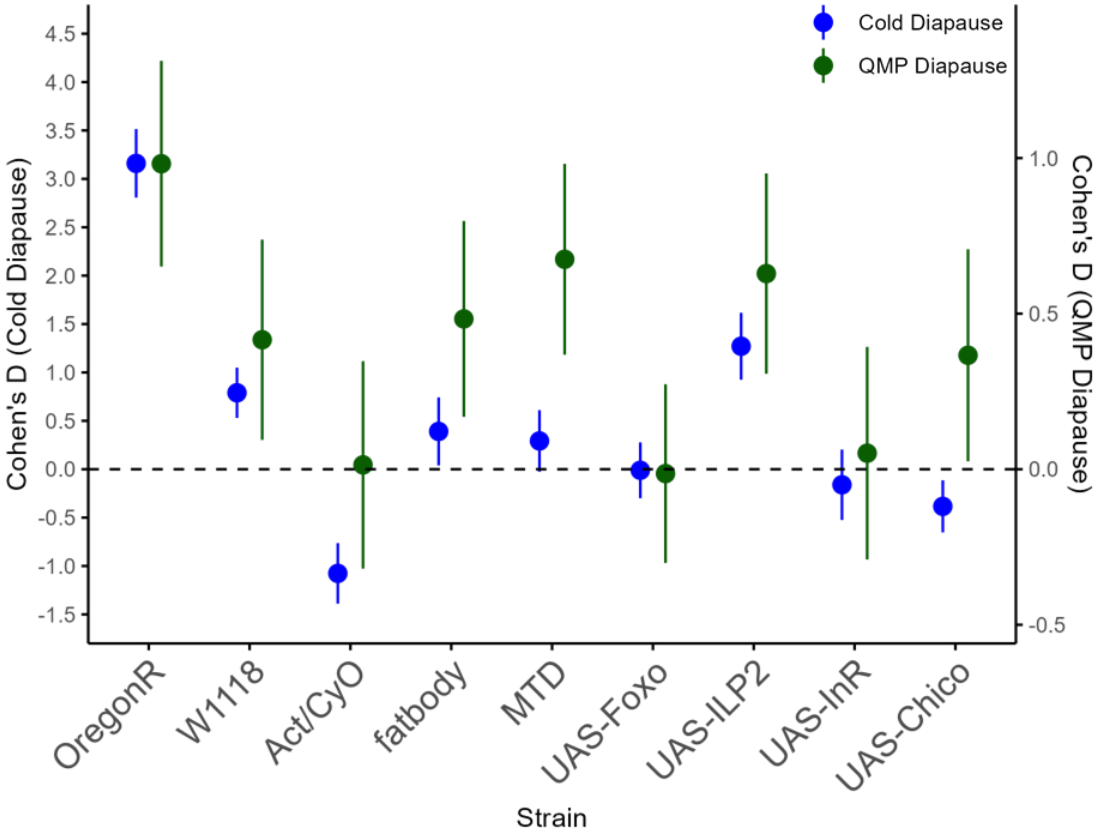


Figure 6.8 – Showing the different effect sizes of cold and QMP induced diapause for different parental strains of *D. melanogaster* used for UAS-Gal4 crosses, as well as two wild-types strains for reference. The x-axis shows nine different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The dashed line represents an effect score of zero (no difference between control and treatment. The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively. The various parental lines have different sensitivities to diapause, as well as different degrees of correlation between diapauses.

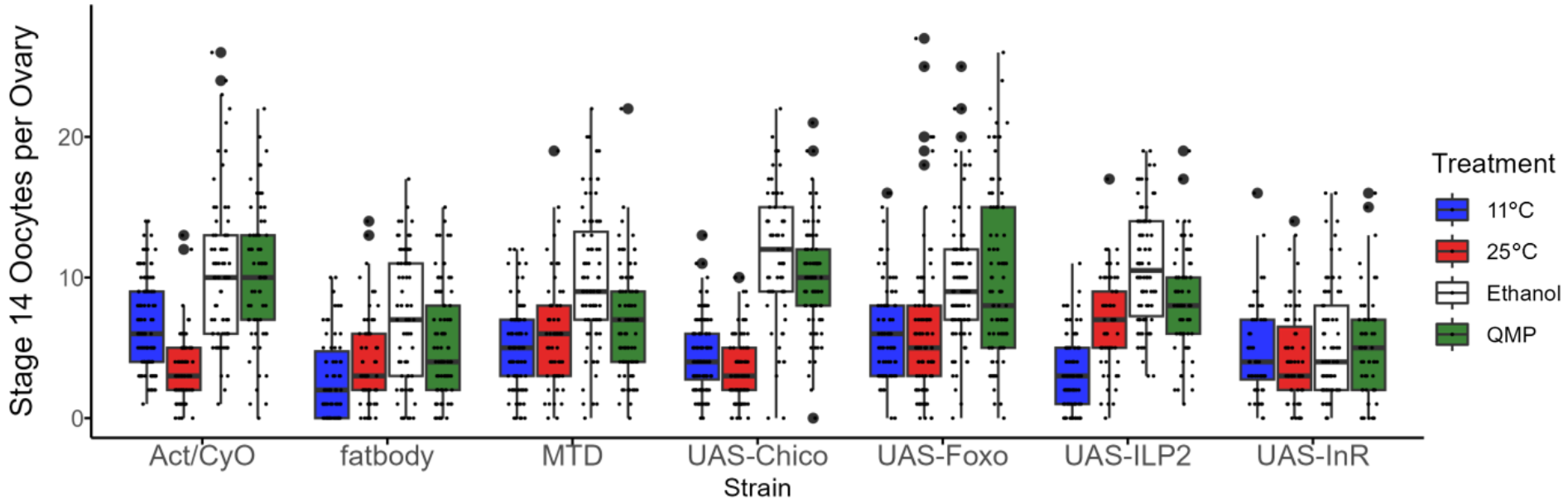


Figure 6.9 – Showing boxplots of the ovary activation of different strains of parental lines of *D. melanogaster* to different diapause conditions. The x-axis shows different strains of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.3.4.2 Over-expressing ILP2 in somatic cells is lethal

D. melanogaster has several insulin-like-proteins, produced in various tissues, primarily in the brain. The insulin-encoding gene ILP2 had been identified as important in the somatic response to cold diapause in fruit flies (351). This gene was investigated for its role in QMP and cold diapause.

Four crosses total were attempted: ILP2xsev as a background control; ILP2xfat for fatbody localisation; ILP2xact for systemic expression; and ILP2xMTD for localisation to the germline of the ovary. However, all crosses except ILP2xsev failed to produce any adult offspring, and never beyond the early larval stages. Presumably this was due to the lethal effects of these crosses in early development.

Insulin signalling is an important hormonal signalling pathway in *D. melanogaster* development, and so it is not altogether surprising that the over expression of such an important aspect of the signalling pathway results in lethal effects.

The ILP2 samples still provide a useful confirmation of the control however, as we can compare the effect of the cross on the responsiveness of the organism to QMP and cold diapause.

As we can see in Figure 6.10, overexpressing ILP2 in the eye produced no difference in effect size for either form of reproductive repression. This provides an important validation of the use of the *Sevenless* gene as a negative control.

Additionally, as can be seen in the raw data in Figure 6.11, we see an overall greater ovary activation for ILP2xsev, versus the ovary activity of UAS-ILP2 (GLM: $F=131.28$, $df=711$, $P<0.001$), highlighting that the cross had successfully resulted in a stronger genetic health of the line, but not in a way which disrupts the effect size of diapause.

This latter observation is important, as an alternative hypothesis for the explanation of the correlation of cold and QMP-mediated diapause is that the smaller the ovaries the smaller the effect size. This is because the measure used for investigating ovary size is via counting the developed eggs of the ovary, whereas the correlation is between Cohen's D effect sizes, a similar correlation also exists between effect sizes as absolute size of ovary in the given strain. The correlation seen previously in Figure 6.4 of wild-types might simply be that the absolute size of ovaries is lower, and so any effect size measured is hampered by the small absolute observations (seen in Figure 6.5).

The increase in ovary size with identical effect size of the ILP2 crosses provides counter-evidence to this hypothesis, as the effect size remains the same with significant differences in absolute size of the ovaries.

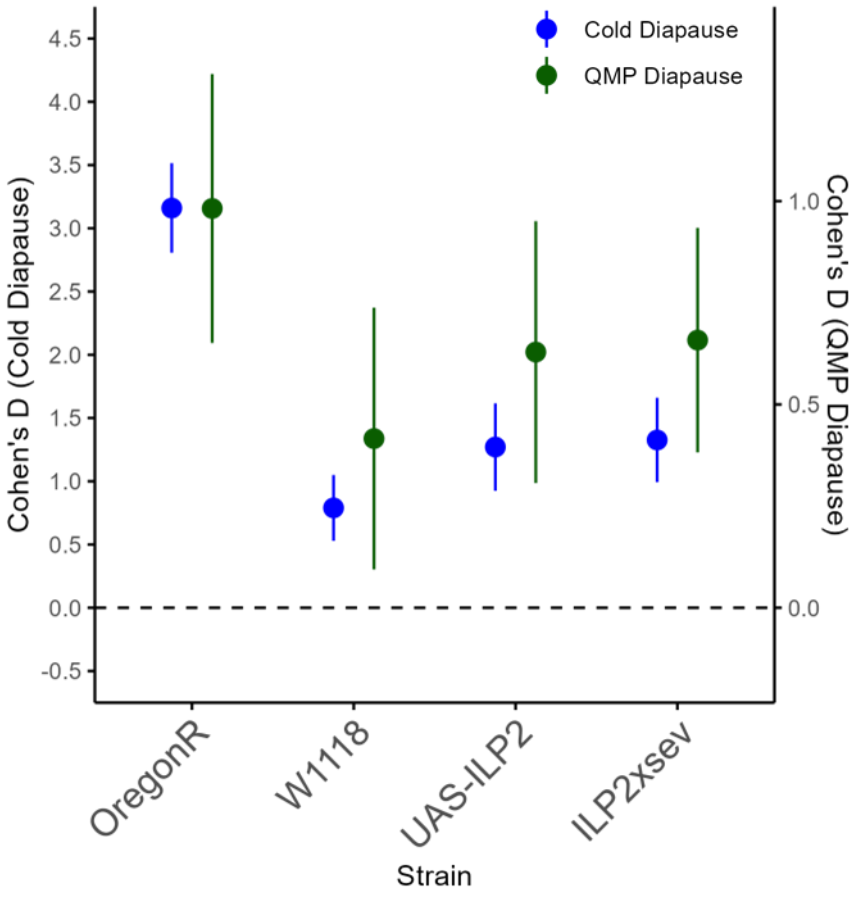


Figure 6.10 – Showing the different effect sizes of cold and QMP induced diapause for the parental line UAS-ILP2, and a UAS-ILP2xSevenless-Gal4 cross of *D. melanogaster* flies, as well as two wild-types strains for reference. The x-axis shows four different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The dashed line represents an effect score of zero (no difference between control and treatment. The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively. Expressing ILP2 in the eye of the fruit fly does not produce any change in its response to cold or QMP diapause.

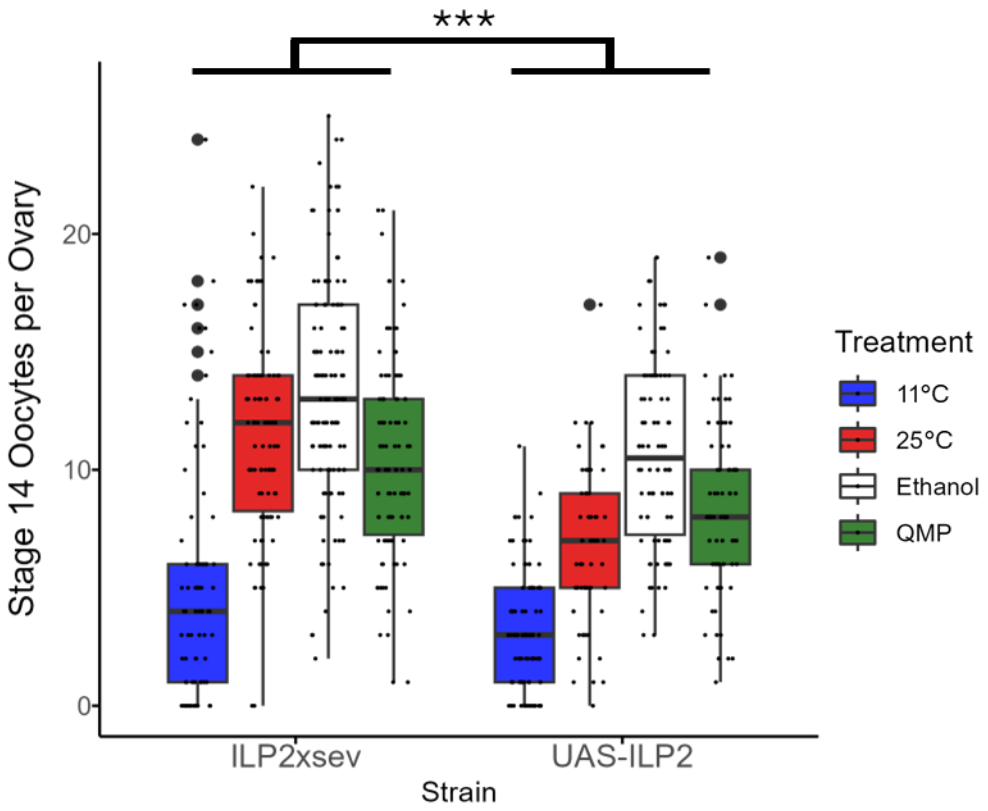


Figure 6.11 – Showing boxplots of the ovary activation of different strains of ILP2xsev and UAS-ILP2 *D. melanogaster* flies to different diapause conditions. The x-axis shows different strains of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.3.4.3 Increased expression of *chico* in the fatbody results in reversal of cold-mediated diapause, but not QMP-mediated diapause

The insulin signalling pathway includes a tyrosine kinase receptor with docking proteins which become activated when the ILP binds to the receptor. In *D. melanogaster,* Chico is one of these docking proteins, and facilitates the downstream elements of insulin signalling (352). By overexpressing chico we should theoretically increase the responsiveness of targets of insulin signalling to insulin peptides (116).

Using the UAS-Gal4 system, *chico* was overexpressed somatically, in the ovary, in the fatbody, and in the eye as a control.

As can be seen in Figure 6.13, the control cross ChicoxSev, which has *chico* overexpressed in the eye, demonstrated a lack of responsiveness to either type of diapause. The parental line UAS-Chico demonstrates a similar lack of responsiveness under cold conditions, so it is possible that the combination of these two backgrounds is inhibitive of diapause generally, making this control unreliable.

Chicoxact, the cross in which Chico is overexpressed globally in the fly, showed a general increase in effect size to both forms of diapause relative to both parental lines. Indeed, this cross seems to have completely mitigated the lack of cold diapause seen in UAS-Chico, and the inverted response to cold diapause seen in Act/CyO-Gal4.

Similarly, with ChicoxMTD, where *chico* is overexpressed in the germ cells of ovary tissue, we see a fairly consistent response to both cold and QMP. In this cross the correlation between both diapause responses matches that of the wild-type W1118 (which is also the background of both lines).

In both of these crosses we see that overexpressing *chico* somatically, and in the ovaries, produces no effect on diapause, and that the crosses simply restore wild-type functionality. The lack of disruption of this overexpression might be a result of the role of Chico in the insulin signalling pathway, whereby over-expressing this gene does not actually augment signalling. Chico acts as a transducer of insulin signalling into the cell, but does not provide potentiation of that pathway, at least under diapause conditions.

This contrasts strongly with the response seen when *chico* is overexpressed in the fatbody (Chicoxfat), where we see an ordinary effect size for QMP-sensitivity but not for cold-diapause, where in fact the response has been reversed. Two conclusions can be drawn from these data:

- That insulin-directed QMP-mediated repression of ovaries in the fruit fly is not mediated via insulin signalling via the fatbody
- That cold-induced diapause is mediated via insulin signalling via the fatbody.

These results strongly imply that the mechanism of action of these two different diapause phenotypes are mediated through different tissues. A possible scheme for this is shown in Figure 6.12. In this model, cold-mediated diapause does not inhibit the ovaries directly, but is mediated via the fatbody (which could occur e.g. by the disruption of vitellogenesis in the fatbody). Disruption to insulin signalling in the ovary therefore does not result in this signal being transduced from the fatbody to the ovary.

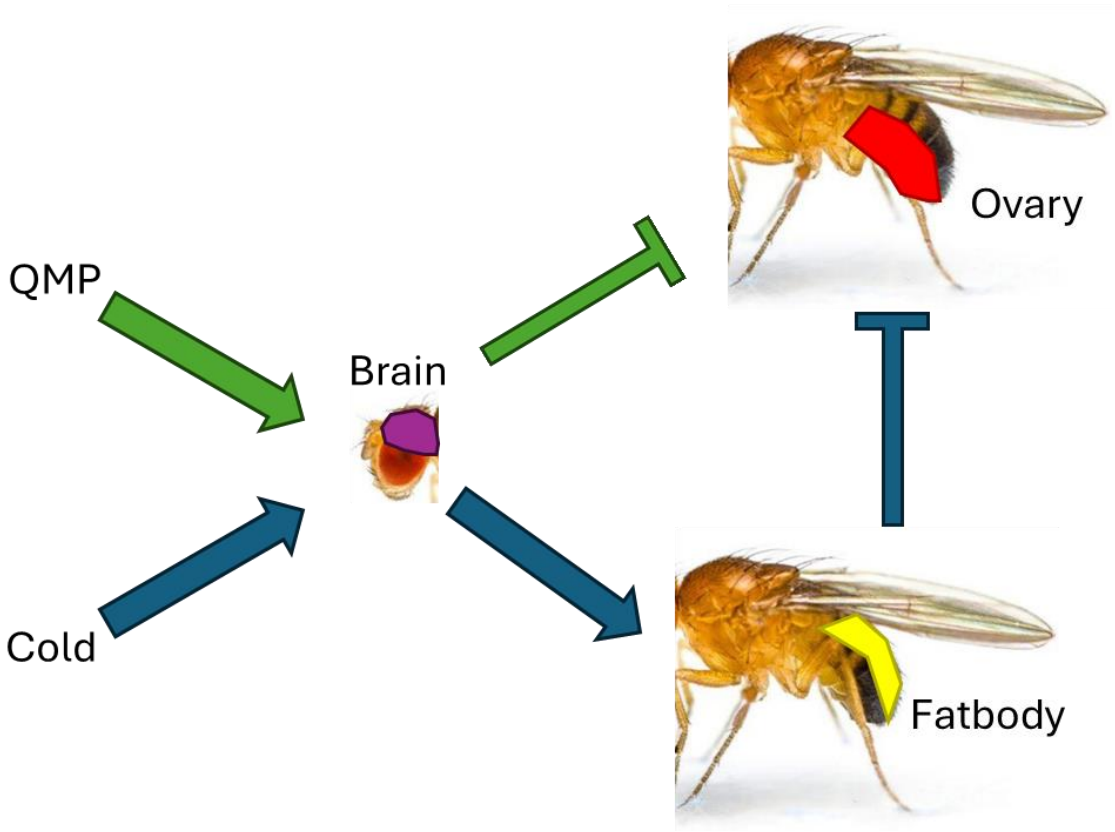


Figure 6.12 – Detailing possible pathways for responses of QMP and Cold-mediated diapauses. QMP and cold conditions both bring about diapause. This simple scheme shows two possible routes that the decision to repress ovaries follow. In the green route we see QMP detected and transduced to the brain, which brings about hormonal changes resulting in the inhibition of ovaries. In the blue we see cold producing the same decision in the brain, but enacting changes in the fatbody via insulin signalling, which then lead on to result in ovary repression from signalling output from the fatbody.

The correlation seen in Figure 6.4 and Figure 6.6 does not contradict this however, as the "decision" to limit reproduction is one of many physiological and behavioural phenotypes which changes in diapause. The decision itself is likely not mediated in the fatbody itself, but rather in the brain in communication with other organs (116). This decision would be undertaken in the brain in both QMP-exposed conditions and cold conditions.

The fact that increased Chico in the fatbody results in a reversal of cold-mediated diapause, not just a mitigation of this phenotype is interesting, and perhaps hints at a threshold effect. I.e. that the amount of Chico present in the fatbody is itself a mechanism by which different physiological phenotypes are managed, and that e.g. after cold-diapause ends, massively increased insulin signalling results in the exit of diapause of the animal for reproductive

purposes. This specific example is unlikely however, as the raw results in Figure 6.14 do not show a recuperation of ovary activity in 11°C conditions, but rather a significantly repressed phenotype in the 25°C conditions.

The raw data does not show any irregularities with ovary repression or activity (Figure 6.14).

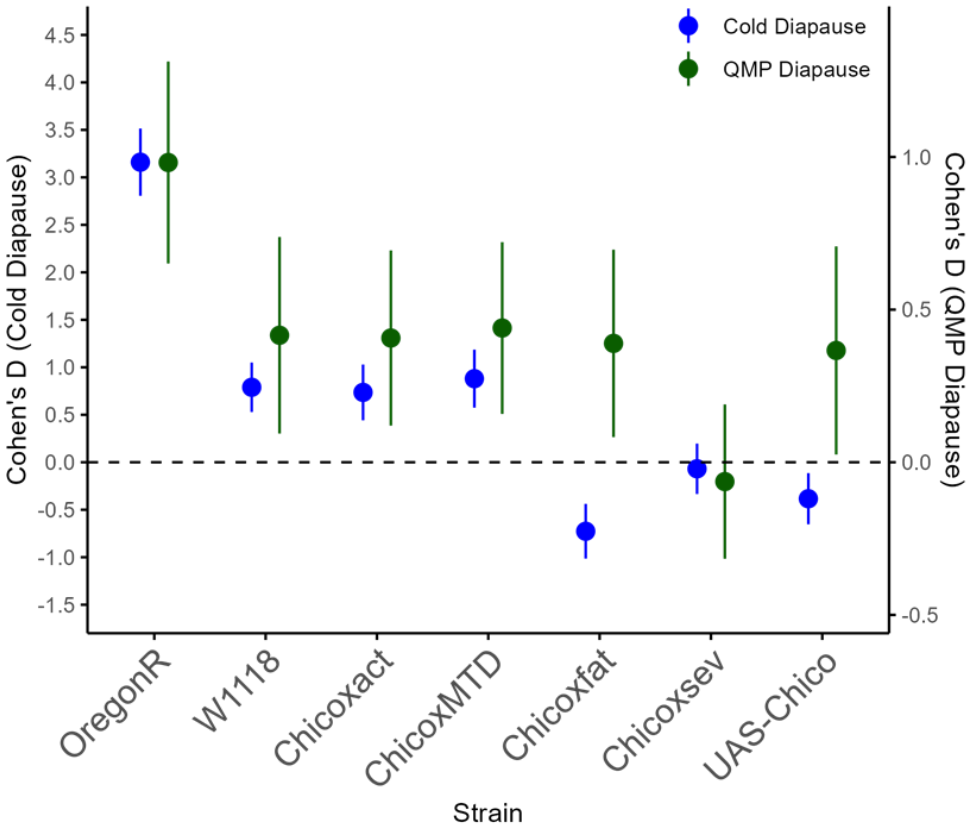


Figure 6.13 – Showing the different effect sizes of cold and QMP induced diapause for UAS-Chico crossed with various Gal-4 lines, and the UAS-Chico parental lines of *D. melanogaster* flies, as well as two wild-types strains for reference. The x-axis shows eight different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The dashed line represents an effect score of zero (no difference between control and treatment. The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively. Expressing Chico systemically or in the ovary has no effect on diapause sensitivity. Expressing Chico in the fatbody inverts the cold-diapause response. Expressing Chico in the eye appears to eliminate QMP-induced diapause.

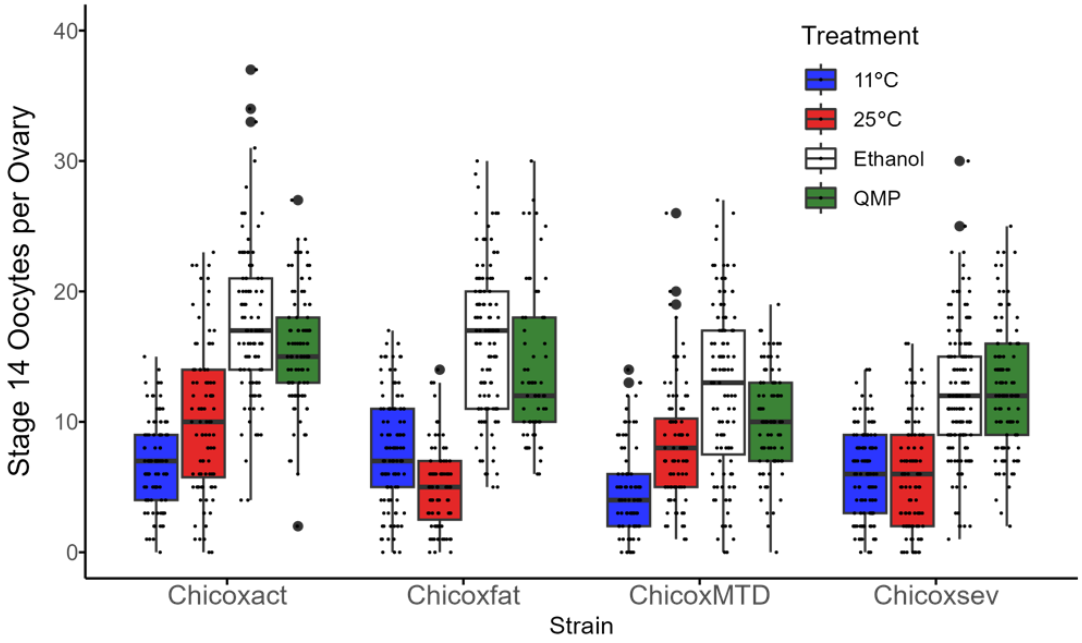


Figure 6.14 – Showing boxplots of the ovary activation of different UAS-Chico crosses with Gal-4 lines of *D. melanogaster* flies under different diapause conditions. The x-axis shows different strains of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.3.4.4 Foxo does not mediate cold or QMP-mediated reproductive repression in *D. melanogaster*

One of the downstream elements of insulin signalling is the transcription factor Foxo. This protein is also a downstream signalling element of glucose transport, and the Epidermal growth factor(EGF) signalling pathway. Insulin signalling typically represses Foxo, so the overexpression of Foxo would theoretically counteract the impact of insulin signalling. (116, 352)

The control Foxoxsev, in which *Foxo* is overexpressed in the eye, shows a marginal decrease in the effect size of QMP, likely due to genetic background effects. For these crosses this control is likely effective.

Foxoxact, in which *Foxo* is overexpressed somatically, was lethal; and Foxoxfat, in which *Foxo* is overexpressed in the fatbody, demonstrated low survivability, and so a low number of samples were generated.

As can be seen in Figure 6.15, the samples of Foxoxfat that were generated were used to investigate the effect of cold diapause, and which did not show the reversal of effect seen when *chico* is overexpressed in the fatbody, likely showing that Foxo is not the mechanism by which cold diapause results in ovary repression in the fruit fly. A pairwise comparison of the raw data shown in Figure 6.16 shows that this is a statistically significant repression (GLM: F=2.103, df=57, P=0.0099).

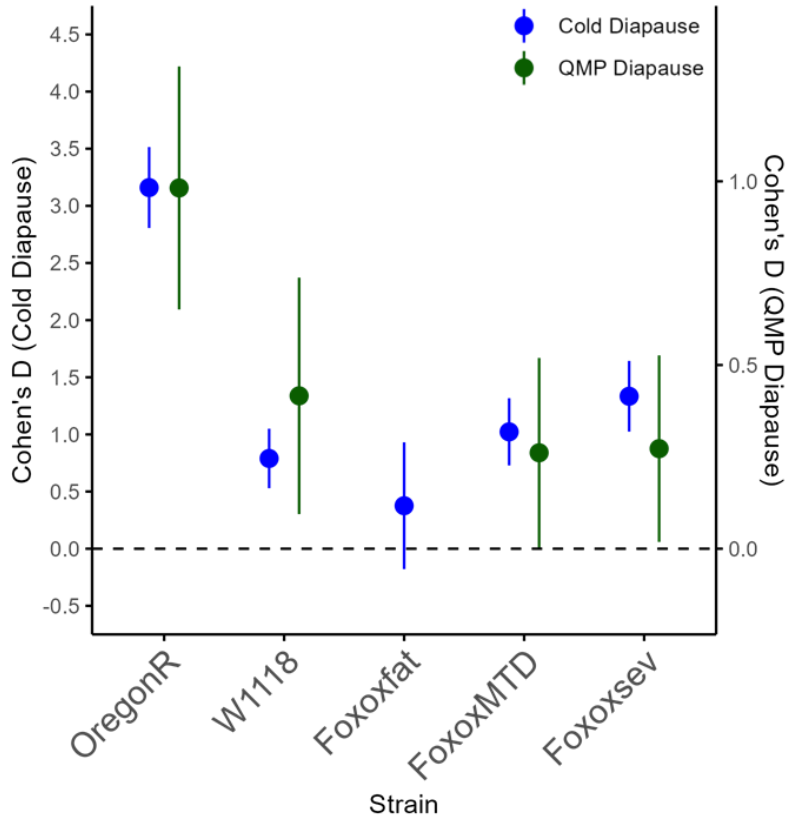


Figure 6.15 – Showing the different effect sizes of cold and QMP induced diapause for UAS-Foxo crossed with three Gal-4 lines (fatbody, ovary, and eye) of *D. melanogaster* flies, as well as two wild-types strains for reference. The x-axis shows five different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The dashed line represents an effect score of zero (no difference between control and treatment. The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively, only cold diapause is present for Foxoxfat. Expressing Foxo in the ovary and eye produces no difference in effect on diapause.

When *Foxo* is overexpressed in the ovaries (FoxoxMTD), there is no difference in effect size for either type of diapause relative to the *Sevenless* control. This shows that Foxo is not involved in the cold or QMP-mediated repression of ovaries.

The raw data shown in Figure 6.16 shows no great irregularities in ovary activity or repression.

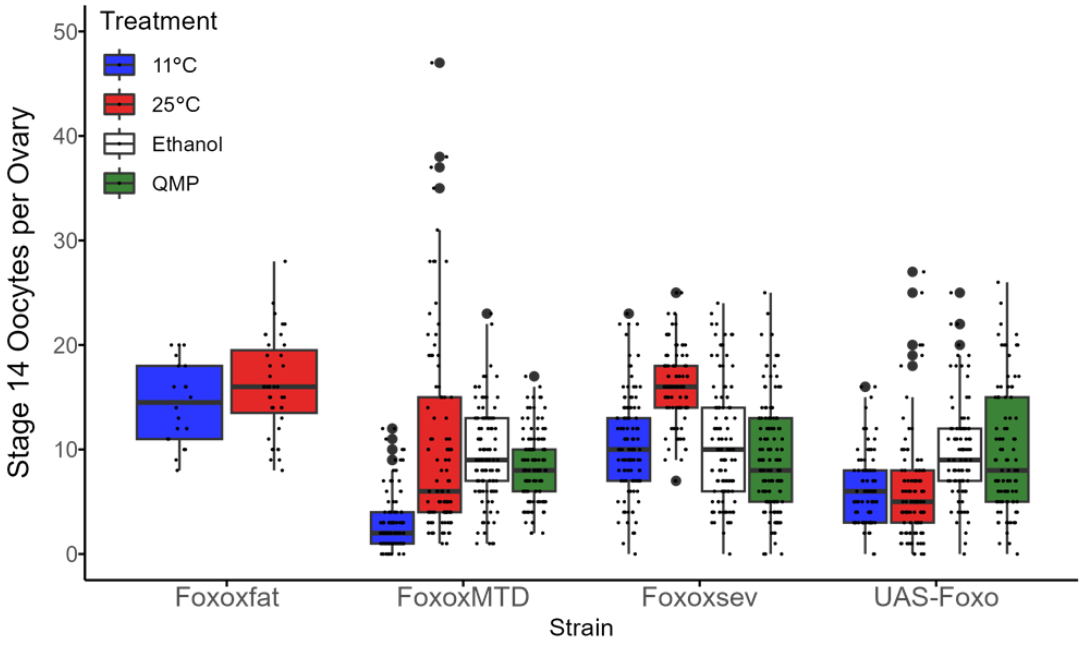


Figure 6.16 – Showing boxplots of the ovary activation of different UAS-Foxo crosses with Gal-4 lines of *D. melanogaster* flies under different diapause conditions. The x-axis shows different strains of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.3.4.5 Expression of InR in fatbody and ovary does not bring about differential effects in cold and QMP-induced diapause

The last insulin signalling protein targeted was the receptor itself InR. This tyrosine kinase receptor forms the first part of the insulin signalling pathway in the tissue receiving the endocrine signal. Overexpressing it in a given tissue therefore should increase the magnitude of any signalling attempt using this pathway.

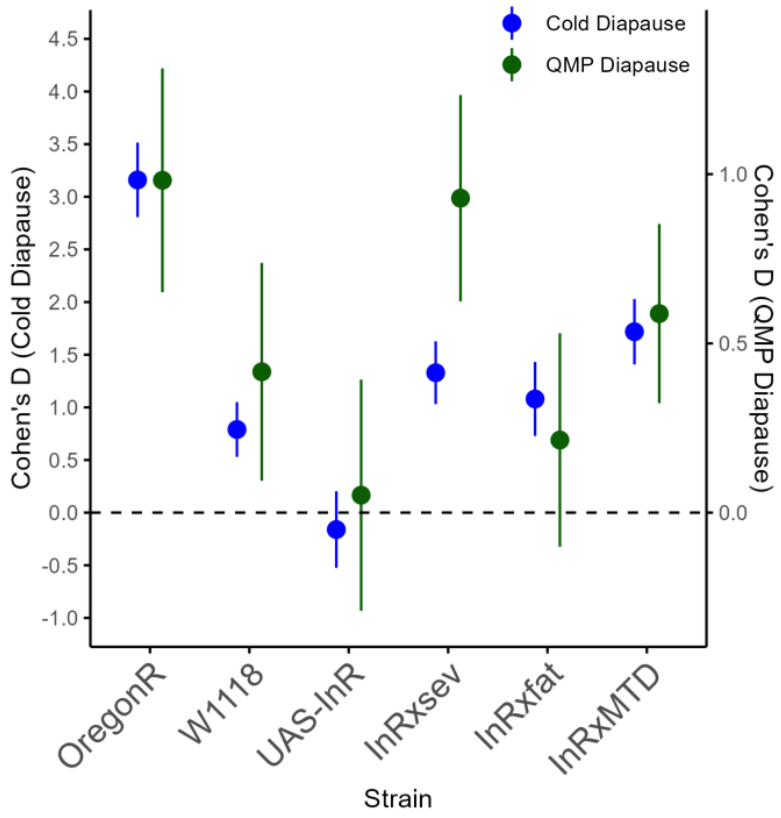


Figure 6.17 – Showing the different effect sizes of cold and QMP induced diapause for UAS-InR crossed with three Gal-4 lines (eye, fatbody, and ovary) of *D. melanogaster* flies, as well as two wild-types strains for reference. The x-axis shows six different strains. The left y-axis shows the Cohen's D effect size of cold-induced diapause (and corresponds to the blue data), while the right y-axis shows the Cohen's D effect size of QMP-induced diapause (and corresponds to the green data). The dashed line represents an effect score of zero (no difference between control and treatment. The effect size is the CohensD on the number of stage 14 vitellogenic oocytes of virgin female flies exposed to 11°C and 25°C for diapause and control respectively for the cold-induced flies; while the QMP induced flies have been exposed to 6.5Qe QMP or ethanol solvent control for diapause and control respectively. Expressing InR in any tissue does not change the sensitivity to cold diapause. Expressing InR in the ovary reduces the effect size of QMP-diapause, while expressing InR in the fatbody reduces it further, when compared to the eye-expressed control.

As can be seen in Figure 6.17, the parental line UAS-InR does not produce any significant effect size in cold or QMP-mediated ovary repression, however the InRxsev control, in which InR is overexpressed in the eye shows a large increase in the effect size of QMP-mediated diapause, but not cold diapause. This is perhaps due to off-target effects in this cross (as sev-Gal4 is known to have some off-target effects involving brain chemistry).

Due to the lack of consistency of the controls in these samples, the confidence of conclusions drawn are less than in investigating previous genes.

It does seem that when expressing InR in the eye, fatbody, and ovary, (sev, fat, and MTD respectively) we see relatively consistent responses to cold diapause. There is a marginal increase in effect size in the ovary when compared to the fatbody in particular.

A more marked difference in effect size of the QMP-response seems to exist, however this may be an artifact of the different axis scales, and more likely just indicates that there is a correlation between the two types of diapause regardless of where InR is over-expressed in the fly.

In conclusion, expression of the InR receptor does not seem to result in any differentiation between the two types of diapause seen in the fruit fly.

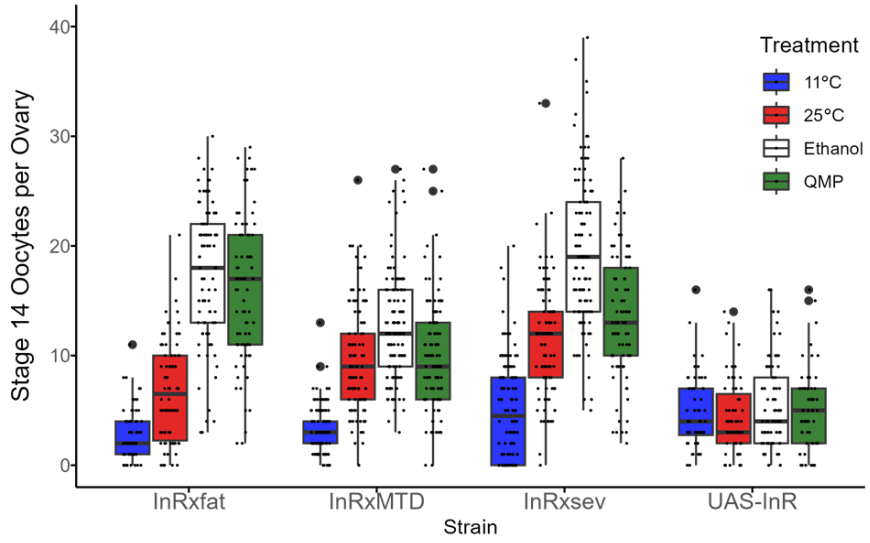


Figure 6.18 – Showing boxplots of the ovary activation of different UAS-InR crosses with Gal-4 lines of *D. melanogaster* flies under different diapause conditions. The x-axis shows different strains of fly, while the y-axis shows the number of stage 14 vitellogenic oocytes per ovary. Each strain shows four diapause conditions: to 11°C and 25°C for diapause and control respectively for the cold-induced diapause; and 6.5Qe QMP or ethanol solvent control for QMP-induced diapause; these are also shown with colour.

6.4 Discussion

The fruit fly is responsive to the honeybee queen pheromone QMP, despite being ~350 million years diverged (3). The sensitivity of this organism to this pheromone, as well as the unusual complexity of QMP relative to other queen pheromones, has resulted in a model being proposed whereby the evolution of QMP to maintain repression of the worker honeybee's reproduction has occurred to such an extent that the pheromone has co-opted a fundamental aspect of invertebrate biology (132).

The Notch signalling pathway has been shown to be important in the honeybee for QMP-mediated reproductive repression (132), but it has also recently been shown that *D. melanogaster* exposed to QMP behave in a manner similar to starvation, and that their insulin signalling matches this too (183).

There are a limited number of hormonal signalling pathways in insects (e.g. ecdysone, insulin, juvenile hormone), and the response of a given organism to one of these pathways must often be contextualised in the fact that these holistically influence the signalling of other pathways (116). As a result, the changes in insulin signalling that have been shown to occur in fruit flies in response to QMP, must be understood not necessarily as a direct reaction to this pheromone. The nature of this signalling must still be investigated further, and with regards to the other possible pathways that exist in order to fully understand this mechanism.

Increasing our understanding of the mechanism of action of QMP-mediated reproductive response in the fruit fly, allows us to better design experiments investigating the role of this pheromone in the honeybee. If a common mechanism of action of this pheromone in these two organisms exist, then we must first elucidate the mechanism in both.
If the mechanism is different, then there are many questions regarding the evolution of QMP, and why it is able to elicit a response in the fruit fly, while if the mechanism is the same, then it allows us to investigate QMP-mediated reproductive constraint in fruit flies with the much more powerful tools available in this organism relative to the honeybee.

6.4.1 Diapause as the proposed mechanism of action of QMP-mediated reproductive repression

In response to adverse environmental conditions, insects possess the ability to limit their metabolism and reproductive physiology in order to devote their bodily resources to survival (198, 259). This is related to maximising their reproductive fitness, as reproducing during adverse conditions is far less likely to successfully produce offspring. Conserving resources until a better opportunity maximises the likelihood of successful reproduction, as well as increasing

the number of possible eggs laid. It is therefore highly advantageous for the organism to possess a plastic response to these adverse conditions.

In the *D. melanogaster*, this reproductive diapause also intersects with longevity, as reproduction, both in the act of mating and laying eggs, reduces lifespan in accordance with the longevity-fecundity trade-off (181). Diapause maximises lifespan for continued reproduction in more favourable reproductive conditions.

That fruit flies possess this plasticity in response to the presence of QMP, as well as the correlation in sensitivity to QMP and cold for induction of diapause, suggests that QMP-mediated reproductive repression in fruit flies may be working through some form of diapause mechanism as proposed in the literature (103, 104).

That the wild-type strains (Figure 6.4) and mutant lines (Figure 6.6) predominantly demonstrated a strong correlation between the effect size of cold and QMP-induced diapause provides evidence that QMP and cold-induced diapauses operate through the same or similar input mechanisms. The differing sensitivity that these strains exhibit towards the cold are representative of natural diversity that exists in the diapause response. The fact that the QMP-induced diapause effects also show broadly the same sensitivities as cold-diapause implies they may work through the same pathways.

For example, if the decision to enter diapause occurs as a result of a combination of various inputs in the brain of the fly (as proposed by (103)), sensitivity to one input may correlate with sensitivity to another input.

The transcription factor Foxo has also been implicated in the cold-diapause response (initially in mosquitoes (353), then subsequently by proxy of 4-EBP in fruit flies (199, 354)). The lack of change in cold-induced or QMP-induced diapause effect size in response to overexpression of Foxo in the ovary strongly implies that QMP is acting via the same mechanism of action as cold diapause, at least as far as insulin signalling has been demonstrated to be involved. If Foxo is involved with only cold diapause, then we should expect to see changes to the responsivity of the flies to cold diapause in conditions of over-expression of Foxo. This is because insulin signalling acts as an inhibitor of Foxo, so overexpression of Foxo should to some degree inhibit the diapause response (116, 352).

These correlative data, both the correlation between cold and QMP-induced diapause effect sizes, and the correlation of these effect sizes in conditions of over-expression of Foxo, strongly imply that the QMP-induced diapause, and the cold-induced diapause, are functioning through

the same or proximate mechanisms. This is particularly in the case of inputs for the decision of diapause-induction.

6.4.2 Differences between QMP and cold-induced diapause

Although there is good evidence for the fact that the diapause is induced by both QMP and cold, there is also evidence that the mechanism by which that diapause is instigated occurs differently.

By over-expressing Chico in the fatbody, the effects of cold and QMP-induced diapause can be uncoupled. QMP-induced diapause remains unchanged, but cold-induced diapause does not only reduce in effect size, but reverses, such that cold conditions significantly increase reproduction.

This finding of Chico results in two different mechanisms by which diapause is mediated. In QMP-mediated diapause, the act of repression of ovaries must not be mediated via the fatbody, whereas in cold-mediated diapause the act of repression must occur via the fatbody. This might not be the direct signalling path as laid out in Figure 6.12, it possibly represents the fact that the cold-response is different to the chemical-response in diapause conditions.

For example, it might be that the overexpression of Chico in cold conditions interferes with metabolic changes in the fatbody which override the repressive effect of diapause on the ovary. These metabolic changes might not occur in chemically-induced diapause conditions, and so the repressive effect in the ovary is unchanged. This would also go some way as to explain why the cold-diapause response was not just mitigated in this fly cross, but reversed in its entirety.

Further investigations into the nature of this signalling pathways and how they have been affected can be continued by looking at specific gene expression levels in this cross in various tissues in order to understand the knock-on effects of Chico overexpression in the fatbody. Attempting to investigate the inverse of this effect, by e.g. expressing RNAi targeting Chico expression in the fatbody and investigating its effect on diapause and QMP-mediated repression of ovaries in the fruit fly. Similarly investigating the effect of the Chicoxfat cross on brain chemistry and signalling, as well as the neuroendocrine pathways involved, such as dopamine and octopamine might reveal any differences in the signalling of these molecules as a result of overexpression of Chico in the fatbody. A metabolomics assay could be investigated, as Chico is a known mediator of carbohydrate metabolism (352), in order to investigate if metabolic changes are responsible for the ovary effects, or perhaps investigating the role of vitellogenesis and its influence by these changes in Chico.

Notably any observation made regarding the effect of Chico in the fatbody are unlikely to occur via the Foxo transcription factor. Insulin signalling represses the activity of Foxo, and so overexpressing this transcription factor should result in the inverse response of that seen with overexpression of Chico. I.e. an increased effect size of cold-diapause, which was not observed.

As a result of the lack of importance of Foxo, the effect of overexpressed Chico in the fatbody on cold diapause is likely to be mediated by one of two other signalling pathways: the glycogen synthesis pathway mediated by Sgg and GS, or the TOR pathway. The TOR pathway is a prime candidate for further investigation, as it is highly conserved and so possibly provides a point of investigation for comparing the similar effects of QMP in fruit flies and honeybees. Indeed, in honeybees it is closely associated with caste determination (355, 356), as well as other effects also seen in fruit flies such as longevity and nutrition (357, 358).

It is also the case that while the effect sizes for the differently induced diapause types correlates strongly, it does not do so absolutely, and several of the parental lines show decoupled responses to cold and diapause.

These data therefore show that although QMP and cold-induced diapause are likely part of the same decision pathway, it is very clear that the application of these diapause decisions are manifested through different tissues and in different ways.

Further investigation into the precise differences in mechanism also present themselves.

First, the potential role of TOR can be investigated in a similar manner to that of the insulin signalling pathways which have been investigated here. By overexpressing specific elements of the TOR signalling pathway, such as Tsc1/2, which should provide an inhibitory effect of the TOR pathway, or Tor itself which should increase the signalling effect; and observing if the same or inverse responses are seen as the Chicoxfat cross on cold-diapause effect.

Similarly, RNAi, which can be used to reduce the expression of a given gene by targeting degradation of the mRNA of a given gene, can be used to target specific elements of this pathway in an inverse pattern to that seen with UAS-Gal4. This can be used to target e.g. *Susi*, or *Pten*, which are inhibitors of PI3K's phosphorylation of PIP2 into PIP3, which should mimic the effect of Chico as a method of reproducing this data.

By targeting Chico, and systemically reducing insulin signalling in this tissue, it may show whether there is an attenuation effect of Chico, and that specific levels of insulin signalling in this tissue produce different effects. In this scheme, a U-shaped response might explain why the over-expression of Chico results in an inversion of the cold-diapause response. By reducing the levels of Chico, it might result in a strengthening of the cold diapause effect (as a linear

response), or it might similarly result in the loss of cold diapause (as a U-shaped response). This might explain why Chico brought about a change in diapause response to cold but not to QMP.

6.4.3 Evolutionary Implications

Although the diapause responses of cold and QMP are manifested through different internal pathways, the similarities that exist between the two forms strongly imply that QMP is instigating reproductive constraint via diapause mechanisms.

This implies that QMP may have evolved in honeybees to take advantage of diapause systems, as suggested by Knapp et al. (103) and Lovegrove et al. (183), who suggested that starvation signalling pathways are possibly responsible for the role of QMP-mediated reproductive repression by virtue of its contribution to the diapause responses that exist across the insects (179, 196, 198, 259).

In *A. mellifera*, as discussed in previous chapters, diapause may have been coopted for the maintenance of reproductive constraint in order to maintain their complex eusocial structure.

The co-option of these mechanisms would also explain why QMP is able to act in fruit flies and other phylogenetically diverse species, as diapause mechanisms are highly conserved in the insect class. In this model, QMP is able to instigate some form of diapause response, but which is independent of the fatbody in this species.

In this way, QMP-mediated reproductive repression in fruit flies is not a result of the same evolutionary pathway that has resulted in its use as a queen pheromone in honeybees, but as a by-product of the co-option of the sub-lethal diapause-inducing effects in honeybees, resulting in latent ovary repression in the fruit fly. I.e. that is that QMP-induced reproductive constraint has come about as a result of sensory exploitation.

6.5 Conclusions

There is a strong correlation between QMP-mediated reproductive repression and cold-induced diapause in fruit flies, implying that QMP is instigating repression via diapause mechanisms.

However, there is also strong evidence that QMP-mediated reproductive repression is not manifested via the fatbody in the manner that cold-induced diapause is manifested, suggesting that the correlation seen is as a result of similar, but not the same mechanisms.

Chico is involved in the cold-diapause response in a manner different to the QMP-diapause, but Foxo is equally involved in both, suggesting that TOR may be more important in the diapause response in the fatbody in response to cold.

6.6 Future Work

Several different avenues for further investigation have been made clear from these results, and several which were considered but not completed due to time constraints.

6.6.1 Further investigation of insulin signalling

The discovery of the importance of Chico in the fatbody for cold-diapause but not QMP-diapause, highlights a potential difference in mechanism of action between cold-induced diapause and QMP-mediated reproductive repression.

Insulin signalling was investigated due to it having been identified in the starvation-like response observed in QMP-exposed fruit flies (183), and specific elements of the insulin pathway were investigated in order to cover as wide a range of these as possible. Various other downstream elements of the insulin pathway may be important to greater or lesser degrees, such as the TOR signalling pathway (358), or Sgg-mediated metabolic effects (359). By utilising the UAS-Gal4 system, specific elements of these pathways can be overexpressed in order to attenuate this pathway. In both pathways there are both inhibitory and activating elements, and so there should opportunity to overexpress these elements to produce both outcomes of inhibiting the pathway or over-expressing it.

Alternatively, RNAi might be useful in order to target those specific elements previously targeted. As mentioned earlier, investigating the specific role of Chico in the fatbody, and how it's able to bring about the reversal of cold-diapause but not QMP-diapause might be a useful set of experiments for understanding the differences between the two types.

By utilising RT-qPCR or RNAseq, the relative gene expression of the various elements of these pathways mentioned previously (e.g. Tor, 4-EBP, Sgg) can be quantified in a manner that would allow for more direct comparison of the differences between the two types of diapause.

6.6.2 Other hormones and their role in diapause

Other signalling pathways might also be of interest, investigating for example the role of juvenile hormone, ecdysteroids, and the biogenic amines dopamine and octopamine. The holistic nature of hormonal signalling in insects would suggest that these pathways would also be involved (116), and investigating the effect of over-expression of various elements might also help to elucidate the mechanisms by which cold and QMP diapause differ.

In order to investigate the neurotransmitters that are also implicated in endocrine signalling in *D. melanogaster*, the use of HPLC to quantify levels of these compounds in the nervous system, particularly the brain has been shown as a viable technique. Ecdysone and juvenile hormone

levels can be investigated via the transcriptional profiles of downstream elements of their signalling, such as Kr-h1 for juvenile hormone (271), or E74 for ecdysone (272, 360). This would allow a more direct look at the precise decision making that underpins the move towards diapause, and any differences between the two types of diapause may be observable in this way.

6.6.3 Validation of the efficacy of the crosses

Observing the gene expression of the target genes via RT-qPCR from the target tissue would work as a validation of the original crosses. In particular this would be useful to identify why the controls were often erratic in their responses, additionally better controls could be developed by backcrossing the parental lines to generate the genetic backgrounds (361).

The localisation of different elements of the pathways under investigation can also be undertaken using HCR as a form of *in situ* hybridisation. This would allow for assessment of the leakiness of the Gal-4 lines, as well as confirmation of the correct localisation of the UAS-lines in the target crosses, this could also be carried out via the use of mCherry crosses to the Gal4 lines, allowing for visualisation of the expression via fluorescence microscopy. It would also help to validate the efficacy of the controls, particularly with how off-target effects might be interfering with the investigations into reproductive physiology.

Chapter 7 General Discussion

7.1 Thesis Overview

The aim of the research conducted throughout this thesis was to make headways into the elucidation of the mechanism of action of the pheromonal mediation by which reproductive repression is brought about in insects, and specifically how this relates to the reproductive constraint of worker castes in eusocial insects. This is particularly important for understanding the role of these pheromones within social organisation more broadly, and how they came to evolve.

The majority of the experiments undertaken were investigating the mechanism of the repressive effect on reproduction of QMP on *A. mellifera* honeybees (96) (Chapter 3), *B. terrestris* bumblebees (209) (Chapter 4) and *D. melanogaster* fruit flies (148) (Chapter 6), but other pheromones were also investigated, such as the honeybee larval pheromones BP and EBO on *A. mellifera* (154, 324) and *D. melanogaster* (Chapter 5), and purported bumblebee queen pheromone nC25 on bumblebees (46) (Chapter 4). The role of quercetin as a QMP blocker (322) was also investigated in *D. melanogaster* and *A. mellifera* (Chapter 5).

Throughout the experiments the possible role of diapause as a mechanism which may have been coopted for QMP-mediated reproductive constraint became more evident. As a result fruit flies, in which diapause is easily inducible, were used to investigate the differences between QMP and cold-induced diapause in this species, in order to infer mechanisms in the honeybee, detailed in Chapter 6.

By utilising measurements of physiological changes under certain experimental conditions, as well as molecular techniques, this research aimed to elucidate elements of the mechanism of action of QMP in the test species, which also required the establishment of the bumblebee model within this lab context.

7.2 Diapause as a Mechanism of Reproductive Repression

Insects undergo diapause during periods of environmental stress, during which it becomes disadvantageous to reproduce. The organism forgoes reproduction in order to lengthen its lifespan (by virtue of the longevity fecundity trade-off (237)) and to wait for more optimal conditions in which to lay eggs (though there is a limit to this and reproduction continues after some weeks (199)).

In the fruit fly it is likely that QMP-mediated reproductive repression occurs through diapause mechanisms, as has been shown in Chapter 6. The significant correlation between the effect

size of cold diapause and QMP-mediated repression, across both wild-type fly lines and diapause-impaired mutant lines strongly suggests that QMP is operating through the same diapause mechanisms as cold, however the inversion of cold diapause but not QMP-diapause seen by overexpressing Chico in the fat body, also strongly suggests that the effect of this reproductive repression is operating through different tissues for the two responses (as highlighted in Figure 6.12). It is not clear whether this is indicative of two different diapause-effect mechanisms in fruit flies, or whether it simply indicates that the inputs of two different internal mechanisms happen to correlate (which may for example be caused by the interruption of a metabolic process which interferes with a signalling molecule, such as the interrupted dopamine metabolism caused by the W1118 mutation, and this global disruption of brain signalling results in correlative disruption to any brain-mediated somatic response).

The honeybee possesses a form of diapause in the winter bee phenotype, which is characterised by a variety of behavioural and metabolic changes, and which occurs in temperate climates, or in long periods of rain such as in the rainy season of tropical climates, and allows for the colony to lengthen lifespan and conserve resources while no foraging is possible due to larger climatic changes.

In the honeybee it appears that these diapause mechanisms, which are universal mechanisms in the insect class (179) , have been coopted into the eusocial system and colony lifecycle. This would also explain why *D. melanogaster* is susceptible to the honeybee QMP. If QMP is able to induce diapause by coopting some highly conserved element of diapause induction, then it would be able to induce this very broadly across the insects.

That honeybees seem to possess a similar starvation response to *D. melanogaster* in the presence of QMP (as shown in section 3.3.2) is possibly an indication of similar mechanisms by which QMP is able to bring about reproductive repression in these species, as the fruit fly also possesses a starvation-like response in the presence of QMP (183). It is eminently possible that the QMP-induced diapause seen in Chapter 6, represents a starvation-like diapause, and that this is the same internal mechanism which produces the same reproductive repression in honeybee workers.

Notably the mechanism of action of QMP is not universal in the insects (e.g. recent work showing that the yellowjacket wasp *Vespula vulgaris* does not have its ovaries repressed in the presence of QMP (133)). This can also be explained in this model by virtue of the changing needs of seasonal diapause in the various eusocial and primitively eusocial species and their life-cycles. E.g. *V. vulgaris* uses an annual lifecycle approach in which foundresses are produced in the latter part of the season and diapause overwinter before founding in the spring; this

lifecycle necessitates the rewiring of the diapause response towards overwintering in a manner closely tied to its lifecycle.

The fact that *B. terrestris* is also reportedly susceptible to QMP (though this was not able to be reproduced in this thesis) is a counter-argument to this model on the surface, as it has the same lifecycle as the common yellowjacket, however *B. terrestris* is far more closely related to *A. mellifera* than *V. vulgaris*, and the two bees share a common origin of eusociality. Additionally, although the *B. terrestris* bumblebee has an over-wintering lifecycle in the same manner as the *V. vulgaris* wasp, it is not identical, and the bumblebee does not have to diapause. It only diapauses as a result of lack of forageable material in the local environment, and in environments where there is adequate foraging opportunity the bumblebee queens are able to undergo their normal colony development over the winter (278).

There is also potential counter-evidence to this model in the differences in plasticity in QMP-mediated reproductive repression in fruit flies and honeybees. The lack of adult plasticity in reproductive repression in honeybees (as noted in section 3.3.4) compared to the plasticity noted in fruit flies in response to QMP (as noted in section 6.3.1) indicates that if the models are working through similar mechanisms then the fundamental biology of these mechanisms has shifted between the two species.

Given the role of diapause in the lifecycle of eusocial organisms, it is not unlikely that this biology has shifted, and that the mechanism of action in honeybees of QMP acting as a developmental blocker rather than an initiator of adult-diapause responses. This is not necessarily in contradiction with the model of diapause being the chief mediator of this response, as the solitary red mason bee *Osmia bicornis* is an example of a bee which utilises ontogenetic diapause to delay its metamorphosis in order to emerge at the optimum point for its life-cycle strategy (362), though this species is also not susceptible to QMP-mediated reproductive constraint (123, 270). It is noted that the reproductive dormancy in fruit flies, and the ontogenetic blocks induced in the mason bee are both called diapause, but perhaps represent fundamentally different biological mechanisms.

7.2.1 Differences in Adult Plasticity of Reproductive Repression in *A. mellifera* and *D. melanogaster*

It is also possible that the difference in fruit fly plasticity with regards to QMP-mediated reproductive repression versus the honeybee response, is not an indication of different fundamental mechanisms, but rather attenuation to increased plasticity in the fly as a result of the vastly different life strategies that exist between these two species. Fruit flies, by necessity,

operate on a very short life-cycle turnover, with potentially a full generation in as little as 11 days (363). The role of adult diapause in *D. melanogaster*, especially starvation linked diapause, relates to the short-term maximisation of reproductive fitness in this species. In the honeybee, whose superorganismal lifecycle takes place over a year or so (190), the necessity for increased plasticity of this system is less important, and so represents far less of an important selection pressure. Notably, the fact that this plasticity does exist in the honeybee when other conditions are present (225), possibly indicates that QMP reflects one element of a diapause signal, which by itself is not strong enough to induce a repressive effect on reproduction. In this model the difference in plasticity between the two species might show that the fruit fly diapause response is simply far more sensitive than the honeybee response.

7.2.2 Stress as a Mechanism of Diapause

As suggested by Knapp et al. (103), the manner in which QMP is able to bring about reproductive constraint in eusocial insects might relate to combined stressors from the environment. Various cues sensed, which individually are not very strong, can combine together to bring about diapause (in this paper called reproductive dormancy). Each signal is individually not enough to bring about reproductive repression because in a natural setting the conditions do not occur in isolation. E.g. in the winter: shorter day lengths, colder temperatures, lack of availability of forageable material, are experienced synchronously, and so if the temperature drops without the other environmental cues (like in a mid-summer cold spell), then it is unlikely that honeybees are in need of entering the winter phenotype.

The role of combined stressors must also be taken into account *in cavea* too, and within the broader context of the organism's natural biology. In Chapter 4 the fact that the conditions *in cavea* for the bumblebee cages were as unstressful as possible, may actually have been a detriment to investigating the activity of QMP in this species. Additionally, in all of the *in cavea* honeybee experiments QMP alone was not able to bring about the total reproductive constraint of workers (between 5-15% of bees would always activate their ovaries even in QMP+ conditions). This is also possibly a result of the lack of natural environmental stressors that a honeybee worker might find in a natural environment.

The role of stressors is not necessarily negative within these contexts either, as a likely mechanism to act as an environmental stressor *in cavea* for honeybees is social policing, whereby the workers eat eggs laid by other workers (129). The lack of opportunity to lay eggs in the cages provided, due to lack of honeycomb, meant that this behavioural phenotype was never able to manifest. In the case of social policing, the lack of this behaviour in queenless

settings is likely a contributing factor, with the lack of QMP, which results in the ovary activation of a subset of the workers.

7.3 Genetic and Strain effects

As can be seen throughout the thesis, a common recurring theme is the impact that strain, or genotype, is able to have on the sensitivity of the organism to a given pheromone.

In Chapter 6, this is seen most clearly with the differing QMP-sensitivities present in the large number of different fly lines used throughout the chapter, however this was also discussed in Chapter 4, as a possible explanation of the significant colony-of-origin effect seen in different experiments, and in Chapter 5, as a possible explanation for the lack of sensitivity to BP and EBO which occurred *in cavea*. It is known that QMP sensitivity differs between different wild strains of *A. mellifera*, and that lines can be selectively bred to have low or high QMP tolerance (364).

The large degree of redundancy that exists in honeybee colonies for the maintenance of eusociality within these contexts (47), allows for large degrees in variation of the sensitivity to each of these mechanisms. In this way, diversity can be maximised, acting as a counter to the genetic bottlenecking that occurs in eusocial species (365).

Notably a genetic mechanism for this high diversity has been shown to exist, via the unusually high recombination rates that occur in eusocial insects broadly (366).

7.4 Honest Signal of Fertility or Reproductive Constraint

A debate in the field of pheromone-mediated reproductive repression of insects, is whether the pheromones have evolved to bring about reproductive repression as a consequence of the need for a reproductive individual to signal their fertility to the non-reproductives (i.e. as an honest signal of fertility), or whether the reproductive forcefully represses the reproduction of the non-reproductive against their best interest (i.e. as a form of forcible reproductive constraint). (167)

In the former, it is important for e.g. a honeybee queen to be able to inform the honeybee workers of her reproductive fitness, as the fitness of the colony as a whole is dependent on the great increase in indirect fitness that is conferred by a highly fertile queen.

However, in the reproductive constraint argument, it is important for the queen as her fertility wanes to continue to repress the reproduction of the workers in order to maximise her own individual fitness. Her repression of the workers is not necessarily in the interest of the workers in this scheme.

It has historically been proposed that honeybees have evolved using queen pheromones as a form of reproductive constraint (367). QMP is thought to have evolved from the simple CHCs of the exoskeleton, with the much greater chemical complexity of the pheromone being attributed to the result of an evolutionary arms race between reproductive constraint from the queen and attempts to overcome this from the workers (183).

Additionally, the wide-acting nature of QMP implies that it has taken advantage of ancestrally fundamental signalling (such as the Notch signalling pathway (132)), and so is able to act on animals in which these pathways are still linked to reproduction.

As seen throughout the honeybee experiments in this thesis, honeybee workers do not universally activate their ovaries. I.e. at least 50% of the bees in any given cage will not activate their ovaries under any experimental conditions, *in cavea* or *in alvo*. The lack of constraint needed for these bees can be understood within the theoretical maximisation of the fitness of the colony as a whole.

If the colony as a whole is modelled as a single organism (as is commonly done with eusocial evolutionary genetics (368)), then once the queen has died, and attempts to rear a new queen have failed, the colony is no longer reproductively active, and the colony now no longer has any degree of reproductive fitness. In order to regain that fitness, a subset of workers activate their ovaries and drones are reared. This maximises the possible fitness of the colony by providing males which have the capacity to mate with queens from other colonies. The eusocial structure still exists, as there is still reproductive division of labour. As a result of the haplodiploid nature of the species, the degree of indirect fitness for the non-reproducing workers through the offspring of their sisters is such that it still advantageous for them not to activate their ovaries.

That honeybee workers are unable to have their activated ovaries repressed after exposure to QMP also fits within this model, as it is indicative of the fact that a queen appearing in a hive after the first failed attempt to rear a new queen internally, will not be a sister of the workers in the hive. It is therefore not in the reproductive interest of the original colony's workers to rear the young of the new queen.

The interest of the queen, in forcibly constraining the workers from activating their ovaries beyond that which is in the interest of the workers, must therefore be balanced with the understanding that if she is infertile, or possessing of reduced fertility, it is in her own interest for the workers to behave in a queenless manner and to replace her, as the colony's survival as a whole can only be safeguarded if they replace the queen while there are still young larvae present (so that they can be moved to a queen cell for queen rearing). The range of time

during which it is in her interest to repress the workers as her fertility decreases is therefore relatively narrow.

Therefore, it seems that regardless of which of these two hypotheses best explains the evolution of pheromonally-mediated reproductive constraint, the current state of the organism suggests that QMP is used primarily as an honest signal of fertility for the workers, and the colony as a whole, to understand when their indirect reproductive fitness is best represented by the current queen.

7.5 Reproducibility of Published Works

Throughout this thesis there were several instances where specific findings in the literature were unable to be reproduced, adding to disagreements which are evident in the literature, as well as providing points of contention to work which had otherwise not been reproduced outside of the original lab context in which those findings were first observed.

Given how a core tenet of the scientific method is the necessity of reproducing published data, this represents a not-insignificant issue with the strength of the conclusions presented by the original works and produced difficulties in generating conclusions in this document.

7.5.1 Failure to Reproduce Published Works

In those cases where an experiment can't be reproduced with different methodologies to the original papers, we see a potential elucidation of the mechanism, such as the data presented in section 3.3.4.1 showing that QMP alone cannot bring about repression of already-activated ovaries, despite published conclusions that this phenotype can be reversed by a queened setting (225). The differences in the findings of the original paper and the work presented here can reasonably be taken to be an indication that QMP is either not able to induce the repression of active ovaries, or that is only able to do so in combination with other factors not included (such as the other queen pheromones, presence of brood, or presence of already-repressed workers, all of which are present in the original paper). However, this conclusion could only be made clear if we were able to reproduce the findings of the original paper. In this instance, this was not possible due to practical reasons, but the difference in findings between the two lab contexts are eminently explainable biologically.

On the other hand, in several instances the original findings could not be replicated with identical or very similar methodologies, such as the work presented in sections 4.3.2 (Bumblebee queen pheromone exposures) and 5.3.1 (BP and EBO exposure in honeybees).

In the latter section, detailing the responsiveness of honeybee worker reproduction to BP and EBO larval pheromones, the original methodologies as well as several elements of these methodologies were investigated for their reproducibility. In all cases, the very strong conclusions of the original work were unable to be reproduced.

The role of BP and EBO as essential elements in the maintenance of reproductive constraint in *A. mellifera* is considered common knowledge in this field of biology. This is despite the lack of published reproducibility. The findings published by Arnold (315), Le Conte (324), and Mohammedi (158), all conducted in the same laboratory context, are over 25 years old and since publication have never been confirmed outside of this context.

The lack of reproducibility is not necessarily a reflection of the truth of the original findings, but rather indicates that the biology we are attempting to observe is not as clear-cut as the original findings suggest. With this result in particular, we can compare to the universal response demonstrated for QMP-mediated reproductive repression, which is easily demonstrated in a wide-array of different lab contexts (94, 96, 105, 132, 148, 266, 369). The lack of ease of reproducibility of BP and EBO's ability to induce repression of worker reproduction implies that these pheromones are not essential as mediators of reproductive constrain, which fits into our larger understanding of the redundancy of these types of pheromones in inducing reproductive constraint (47). The many redundant pheromones which allow workers to perceive the fertility of the queen increase the strength of the queen's ability to reproductively repress the workers, and therefore increase the stability of the colony as a whole; thus aiding in the maintenance of the superorganism. QMP is likely the most important of these pheromones, but the relative importance of these two larval pheromone mixtures, BP and EBO, could be interpreted as less important due to its lack of reproducibility. It is particularly disappointing, as in both the original papers, and in the wider scientific understanding as a whole, the importance attributed to larval pheromones is often on par with that of queen pheromones, or even given greater importance. The lack of reproducibility is in direct conflict with these assessments, and that this could have gone 25 years without confirmation is a demonstration of the failure of the scientific process.

Additionally, the difficulty with elucidating queen pheromones in bumblebees, as evidenced by the disagreements between Van Oystaeyen et al. (46), Holman (88), and Amsalem et al. (282), demonstrate the importance of publishing conflicting results. The research conducted in this thesis suggests that both CHC-based queen pheromones and QMP are either very context dependent for their induction of reproductive constraint in *B. terrestris* bumblebees, only producing repression in young colonies (as hypothesised in Chapter 4); or that the choice of

measurement for reproductive activity is essential for observing any of these effects, as shown by Princen et al. (128, 288), who showed that eggs-laid is a better measure for investigating pheromone effect on reproductive repression than Hess-score related measures.

In any case, the lack of reproducibility demonstrated in this thesis on this topic, is another indicator that the biology may be more complex than at first glance, particularly with regards to the importance of queen pheromones within a species. The ease with which QMP's ability to induce reproductive repression in honeybee workers is observed, contrasts strongly with the difficulty in reproducing the published effects of nC25, or of QMP, on bumblebee reproduction. Potentially highlighting that these mechanisms are less fundamental in bumblebees than in honeybees, or that QMP is particularly unique in its importance as a queen pheromone.

7.5.2 Successful Reproduction of Published Works

A demonstration of the importance of reproducibility in the scientific method is also shown in this thesis. It is shown here that QMP is indeed able to induce reproductive repression of *D. melanogaster*, in a dose dependent manner (section 5.3.2.1) (148, 265), and that cold diapause sensitivity is strain dependent in fruit flies (section 6.3.2) (199). This latter finding was expanded here to include investigations into the role of QMP in this species with regards to diapause mechanisms.

Additionally, the importance of confirming one finding published in the literature against another finding, is also demonstrated here in section 5.3.2.3, where the contradictory conclusions of Lovegrove et al. (134), and Velasque et al. (135, 321), with regards to the ability of nC25 to induce reproductive repression in fruit flies. By utilising the methodology of Velasque et al. we demonstrated that the conclusions found by Lovegrove are likely to be correct. Though there is no clear biological indication to explain the results produced by Velasque et al., this clarification will hopefully lead to further investigation by all groups involved, in order to resolve this.

(321)to confirm.

Chapter 8 Appendices

8.1 RT-qPCR primers

Table 15 – Showing the primer sequences and product sizes for the precocious forager experiments in 3.3.4.3.

Gene	Forward Primer	Reverse Primer	Product Size
Dop2	AACGATGGGACGATACGG	TGAATGGAGGAACGACAACA	114
GAPDH	TGCACAGACCCGAGTGAATA	CGAACTCAATGGAAGCCCTA	103
Ef1-α	TGGCAAGTCTACCACCACTG	ATTTCCTGGGCTTCCTTCTC	93
For	CGACAATGCTTCCAAACCA	AGTCTCCTCGGGCAAATTCT	108
Rps8	GGCATAAGAGGCGAGCAA	CGTTGAGGTCCAAGTTTCGT	103
Rpl32	TTCGTCACCAGAGTGATCGT	GGGCATCAAATATTGTCCCTTA	107
Rps18	GATTCCCGATTGGTTTTTGA	CATTCGTTCCAAATCTTCACG	109

Table 16 – Showing the primer sequences and product sizes for the gene targets of the order of hormone signalling in response to QMP experiment, in section 3.3.5.

Gene	Forward Primer	Reverse Primer	Product Size
DDI1	CCAGAGACATTTGGATCAGTTGTT	GCATCGGACATTATAGTTGATTGTG	110
Lily	GATCCAGCACGATTCAACTC	CCGTCTTCACCGTAATCTCTT	93
Puff	TGAAAGTCGTTATGCGGAAGT	CTGATGTGCTCGTCCTCGTA	112
Red	ATGGGGGTAGTCCAGATGAA	CCTACACCACCTACATCCATTG	85
Shell	CCAATCACGCCAAAAGAAAT	AAATCATCACAGCCTCACAGG	106
Trev	ATCTCGGACTCGTTCTCCAT	CCTGGTAAGTAAGACAAAGGTGTT	114
Unk1	CGCTTTACGGACGTGTTAGC	AAGCAACATGCACACTTCCA	225
Zinc1	GCCGTATGCTTGCGTGTA	AAGATTAGATGCGGACTCACG	99
Zinc2	AACCAAGAAATAGCCCTAAACG	TCAGAAATGAATGTGTCTCCAG	118
Rpn2	CGCCTGTAATGGAAACTGAAA	ACACGTTCTTGTTGCTCACG	103
Mrpl44	GCATGGTTGCATATTGGAACA	CGCTCCCACAAGTGCTAGA	99
4-EBP	ACCCCTGGTGGAACACTTTT	GGCATATTTCGTGGTGGTGT	119
bHLH2	GGGAAGCGGGATCAAGATA	AGTCTGGGCGAGGAGATGTA	90
Her	ACCACCACCGTAGCATCATC	ACTTTGGGGAGGCGTGTAA	90
Kr-h1	CTTGATTTGGCGATTCCTGT	TTTGAGAGGCAATGAGACCA	111
Numb	TAGAGACGGCACGACCAGAC	CAGCAAAAGCACATCCCACT	112
PMP34	ACAAAGGAATGGAAGCCAAA	GGAAATTTACACGGGGAACA	188
Rps8	ACGAAACTTGGACCTCAACG	CACTCAGATCCCCATGAAAAA	107
Rpl1	GGTTCGCCCTTAACAACG	CGTTCCAAACAAACAGCACT	119

8.2 Pairwise Test Comparisons for Figure 3.1

Table 17 – Showing post-hoc pairwise test results from **Figure 3.1**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol CBF - Ethanol Fondant	-0.560	0.190	Inf	-2.939	0.0173
Ethanol CBF - QMP CBF	-1.018	0.270	Inf	-3.774	0.0009
Ethanol CBF - QMP Fondant	-1.365	0.204	Inf	-6.682	<.0001
Ethanol Fondant - QMP CBF	-0.458	0.229	Inf	-2.000	0.1880
Ethanol Fondant - QMP Fondant	-0.805	0.146	Inf	-5.513	<.0001
QMP CBF - QMP Fondant	-0.347	0.240	Inf	-1.449	0.4687

8.3 Pairwise Test Comparisons for Figure 3.4

Table 18 – Showing individual day statistical tests for **Figure 3.4A**. A mixture of F and LRT tests depending on distribution

Pairwise Comparison	Df	Residual deviance	P
Day 1	60	9.33	0.9872
Day 2	60	7.32	0.9086
Day 3	60	11.78	0.03983
Day 4	60	7.28	0.01142
Day 5	60	4.33	<0.0001
Day 6	60	5.05	0.0007
Day 7	60	3.60	<0.0001
Day 8	60	4.45	0.02277
Day 9	59	2.59	0.1644
Day 10	55	5.07	0.2096

Table 19 – Showing individual day statistical tests for **Figure 3.4B**. A mixture of F and LRT tests depending on distribution

Pairwise Comparison	Df	Residual deviance	P
Day 1	50	1.24	0.1036
Day 2	50	18.094	0.04535
Day 3	50	23.122	0.09071
Day 4	50	4.0472	<0.0001
Day 5	50	4.9730	0.01071
Day 6	50	4.3917	0.000162
Day 7	50	6.0269	0.1334
Day 8	50	6.3326	0.02795
Day 9	50	4.2565	0.02682
Day 10	42	4.2541	0.7066

8.4 Cohen's D results for Figure 3.4

Table 20 – Showing the CohensD estimates for **Figure 3.4A**

Day	Cohen's D Estimate	95% CI Lower	95% CI Upper
Day1	0.0101	-0.5004	0.5206
Day2	-0.0294	-0.5399	0.4811
Day3	-0.5309	-1.0502	-0.0116
Day4	-0.6362	-1.1593	-0.1131
Day5	-1.0836	-1.6299	-0.5373
Day6	-0.9130	-1.4492	-0.3768
Day7	-1.2516	-1.8093	-0.6938
Day8	-0.5965	-1.1181	-0.0749
Day9	-0.3595	-0.8794	0.1604
Day10	-0.3389	-0.8776	0.1998

Table 21 – Showing the Cohen's D estimates for **Figure 3.4B**

Day	Cohen's D Estimate	95% CI Lower	95% CI Upper
Day1	-0.4598	-1.0242	0.1045
Day2	-0.6030	-1.1726	-0.0334
Day3	-0.4923	-1.0577	0.0732
Day4	-1.6182	-2.2600	-0.9764
Day5	-0.7354	-1.3110	-0.1598
Day6	-1.1316	-1.7317	-0.5316
Day7	-0.4232	-0.9865	0.1401
Day8	-0.6279	-1.1985	-0.0573
Day9	-0.6327	-1.2036	-0.0619
Day10	-0.1143	-0.7232	0.4947

8.5 Pairwise Test Comparisons for Figure 3.8

Table 22 – Showing post-hoc pairwise test results from **Figure 3.8**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP	-0.440	0.134	Inf	-3.275	0.0030
Ethanol - QMP10	-0.027	0.124	Inf	-0.218	0.9742
QMP - QMP10	0.413	0.139	Inf	2.975	0.0082

8.6 Pairwise Test Comparisons for Figure 3.9

Table 23 – Showing post-hoc pairwise test results from **Figure 3.9**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP	-0.970	0.507	Inf	-1.914	0.1346
Ethanol - QMPatDay10	-1.082	0.481	Inf	-2.248	0.0633
QMP - QMPatDay10	-0.112	0.521	Inf	-0.214	0.9750

8.7 Pairwise Test Comparisons for Figure 3.11

Table 24 – Showing post-hoc pairwise test results from **Figure 3.11**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP	-0.8036	0.191	Inf	-4.200	0.0001
Ethanol - QMP28	0.0679	0.177	Inf	0.383	0.9224
QMP - QMP28	0.8715	0.197	Inf	4.428	<.0001

8.8 Pairwise Test comparisons for Figure 3.12

Table 25 – Showing post-hoc pairwise test results from **Figure 3.12**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP	0.957	1.017	Inf	0.941	0.6142
Ethanol - QMPatDay28	-0.497	0.895	Inf	-0.555	0.8437
QMP - QMPatDay28	-1.454	1.014	Inf	-1.435	0.3230

8.9 Pairwise Test Comparisons for Figure 3.5

Table 26 – Showing post-hoc pairwise test results from **Figure 3.5**

contrast	estimate	SE	df	z.ratio	p.value
Fondant - FondantOnly	-0.258	0.193	Inf	-1.337	0.3743
Fondant - PollenOnly	-4.482	0.164	Inf	-27.333	<.0001
FondantOnly - PollenOnly	-4.223	0.139	Inf	-30.353	<.0001

8.10 Pairwise Test Comparisons for Figure 3.14

Table 27 – Showing post-hoc pairwise test results from **Figure 3.14A**

contrast	estimate	SE	df	t.ratio	p.value
Nurse - Guard	0.1843	0.212	34	0.869	0.9748
Nurse - Forager	0.3250	0.212	34	1.533	0.7236
Nurse - Ethanol Inactive	0.1953	0.222	34	0.879	0.9734
Nurse - Ethanol Active	0.6327	0.212	34	2.985	0.0700
Nurse - QMP Inactive	0.4672	0.212	34	2.204	0.3199
Nurse - QMP Active	0.4130	0.212	34	1.948	0.4645
Guard - Forager	0.1407	0.212	34	0.664	0.9938
Guard - Ethanol Inactive	0.0111	0.222	34	0.050	1.0000
Guard - Ethanol Active	0.4484	0.212	34	2.115	0.3669
Guard - QMP Inactive	0.2830	0.212	34	1.335	0.8308
Guard - QMP Active	0.2287	0.212	34	1.079	0.9301
Forager - Ethanol Inactive	-0.1296	0.222	34	-0.583	0.9969
Forager - Ethanol Active	0.3078	0.212	34	1.452	0.7701
Forager - QMP Inactive	0.1423	0.212	34	0.671	0.9934
Forager - QMP Active	0.0880	0.212	34	0.415	0.9995
Ethanol Inactive - Ethanol Active	0.4374	0.222	34	1.967	0.4528
Ethanol Inactive - QMP Inactive	0.2719	0.222	34	1.223	0.8803
Ethanol Inactive - QMP Active	0.2176	0.222	34	0.979	0.9552
Ethanol Active - QMP Inactive	-0.1655	0.212	34	-0.781	0.9853
Ethanol Active - QMP Active	-0.2198	0.212	34	-1.037	0.9416
QMP Inactive - QMP Active	-0.0543	0.212	34	-0.256	1.0000

Table 28 – Showing post-hoc pairwise test results from **Figure 3.14B**

contrast	estimate	SE	df	t.ratio	p.value
Nurse - Guard	0.18204	0.185	34	0.982	0.9545
Nurse - Forager	0.17138	0.185	34	0.925	0.9659
Nurse - Ethanol Inactive	0.17339	0.194	34	0.892	0.9713
Nurse - Ethanol Active	0.42470	0.185	34	2.292	0.2771
Nurse - QMP Inactive	0.29396	0.185	34	1.586	0.6916
Nurse - QMP Active	0.29240	0.185	34	1.578	0.6967
Guard - Forager	-0.01066	0.185	34	-0.058	1.0000
Guard - Ethanol Inactive	-0.00866	0.194	34	-0.045	1.0000
Guard - Ethanol Active	0.24266	0.185	34	1.309	0.8428
Guard - QMP Inactive	0.11192	0.185	34	0.604	0.9963
Guard - QMP Active	0.11036	0.185	34	0.595	0.9965
Forager - Ethanol Inactive	0.00201	0.194	34	0.010	1.0000
Forager - Ethanol Active	0.25333	0.185	34	1.367	0.8150
Forager - QMP Inactive	0.12258	0.185	34	0.661	0.9939
Forager - QMP Active	0.12102	0.185	34	0.653	0.9943
Ethanol Inactive - Ethanol Active	0.25132	0.194	34	1.293	0.8504
Ethanol Inactive - QMP Inactive	0.12057	0.194	34	0.620	0.9957
Ethanol Inactive - QMP Active	0.11901	0.194	34	0.612	0.9960
Ethanol Active - QMP Inactive	-0.13075	0.185	34	-0.705	0.9914
Ethanol Active - QMP Active	-0.13230	0.185	34	-0.714	0.9908
QMP Inactive - QMP Active	-0.00156	0.185	34	-0.008	1.0000

8.11 Pairwise Test Comparisons for Figure 3.15

Table 29 – Showing post-hoc pairwise test results from **Figure 3.15B**

contrast	estimate	SE	df	t.ratio	p.value
Nurse - Guard	0.00993	0.127	34	0.078	1.0000
Nurse - Forager	0.03870	0.127	34	0.305	0.9999
Nurse - Ethanol Inactive	0.22940	0.133	34	1.722	0.6067
Nurse - Ethanol Active	0.08411	0.127	34	0.662	0.9938
Nurse - QMP Inactive	0.05474	0.127	34	0.431	0.9994
Nurse - QMP Active	0.12176	0.127	34	0.959	0.9594
Guard - Forager	0.02877	0.127	34	0.226	1.0000
Guard - Ethanol Inactive	0.21948	0.133	34	1.647	0.6537
Guard - Ethanol Active	0.07419	0.127	34	0.584	0.9969
Guard - QMP Inactive	0.04481	0.127	34	0.353	0.9998
Guard - QMP Active	0.11184	0.127	34	0.880	0.9731
Forager - Ethanol Inactive	0.19071	0.133	34	1.432	0.7812
Forager - Ethanol Active	0.04542	0.127	34	0.358	0.9998
Forager - QMP Inactive	0.01604	0.127	34	0.126	1.0000
Forager - QMP Active	0.08307	0.127	34	0.654	0.9942
Ethanol Inactive - Ethanol Active	-0.14529	0.133	34	-1.091	0.9266
Ethanol Inactive - QMP Inactive	-0.17467	0.133	34	-1.311	0.8420
Ethanol Inactive - QMP Active	-0.10764	0.133	34	-0.808	0.9825
Ethanol Active - QMP Inactive	-0.02937	0.127	34	-0.231	1.0000
Ethanol Active - QMP Active	0.03765	0.127	34	0.296	0.9999
QMP Inactive - QMP Active	0.06703	0.127	34	0.528	0.9982

8.12 Pairwise Test Comparisons for Figure 3.17

Table 30 – Showing post-hoc pairwise test results from **Figure 3.17**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP	-0.470	0.110	Inf	-4.285	0.0001
Ethanol - QMP from Day 3	-0.219	0.125	Inf	-1.747	0.2995
Ethanol - QMP from Day 5	-0.419	0.151	Inf	-2.781	0.0278
QMP - QMP from Day 3	0.251	0.129	Inf	1.949	0.2078
QMP - QMP from Day 5	0.051	0.154	Inf	0.331	0.9875
QMP from Day 3 - QMP from Day 5	-0.200	0.165	Inf	-1.212	0.6190

8.13 Pairwise Test Comparisons for Figure 3.18

Table 31 – Showing post-hoc pairwise test results from **Figure 3.18**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP	-0.7534	0.488	Inf	-1.545	0.4107
Ethanol - QMPatDay3	-0.9410	0.504	Inf	-1.866	0.2427
Ethanol - QMPatDay5	-0.7259	0.608	Inf	-1.194	0.6304
QMP - QMPatDay3	-0.1877	0.471	Inf	-0.398	0.9786
QMP - QMPatDay5	0.0274	0.581	Inf	0.047	1.0000
QMPatDay3 - QMPatDay5	0.2151	0.595	Inf	0.362	0.9838

8.14 Pairwise Test Comparisons for Figure 3.21

Table 32 – Showing post-hoc pairwise test results from **Figure 3.21**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - Ethanol at Day 10	-0.0878	0.155	Inf	-0.567	0.9798
Ethanol - Ethanol at Day 6	-0.2400	0.111	Inf	-2.161	0.1946
Ethanol - Ethanol at Day 8	0.1903	0.143	Inf	1.329	0.6733
Ethanol - QMP	-0.5383	0.134	Inf	-4.012	0.0006
Ethanol at Day 10 - Ethanol at Day 6	-0.1522	0.166	Inf	-0.917	0.8906
Ethanol at Day 10 - Ethanol at Day 8	0.2781	0.189	Inf	1.471	0.5815
Ethanol at Day 10 - QMP	-0.4505	0.182	Inf	-2.471	0.0972
Ethanol at Day 6 - Ethanol at Day 8	0.4302	0.155	Inf	2.772	0.0443
Ethanol at Day 6 - QMP	-0.2984	0.147	Inf	-2.032	0.2507
Ethanol at Day 8 - QMP	-0.7286	0.173	Inf	-4.222	0.0002

8.15 Pairwise Test Comparisons for Figure 3.20

Table 33 – Showing post-hoc pairwise test results from **Figure 3.20**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - EthanolatDay10	-0.933	0.642	Inf	-1.452	0.5936
Ethanol - EthanolatDay6	-1.304	0.511	Inf	-2.549	0.0800
Ethanol - EthanolatDay8	-0.725	0.645	Inf	-1.124	0.7938
Ethanol - QMP	-1.640	0.452	Inf	-3.633	0.0026
EthanolatDay10 - EthanolatDay6	-0.371	0.690	Inf	-0.538	0.9833
EthanolatDay10 - EthanolatDay8	0.207	0.794	Inf	0.261	0.9990
EthanolatDay10 - QMP	-0.708	0.646	Inf	-1.095	0.8092
EthanolatDay6 - EthanolatDay8	0.579	0.692	Inf	0.836	0.9194
EthanolatDay6 - QMP	-0.336	0.517	Inf	-0.651	0.9665
EthanolatDay8 - QMP	-0.915	0.649	Inf	-1.410	0.6212

8.16 Pairwise Test Comparisons for Figure 3.6

Table 34 – Showing post-hoc pairwise test results from **Figure 3.6**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol FandP - Ethanol Fondant	-3.9192	0.291	Inf	-13.458	<.0001
Ethanol FandP - QMP FandP	-1.1303	0.156	Inf	-7.224	<.0001
Ethanol FandP - QMP Fondant	-4.0066	0.299	Inf	-13.398	<.0001
Ethanol Fondant - QMP FandP	2.7889	0.289	Inf	9.665	<.0001
Ethanol Fondant - QMP Fondant	-0.0874	0.381	Inf	-0.229	0.9958
QMP FandP - QMP Fondant	-2.8763	0.296	Inf	-9.703	<.0001

8.17 Pairwise Test Comparisons Figure 3.26

Table 35 – Showing post-hoc pairwise test results from **Figure 3.26A**

contrast	estimate	SE	df	t.ratio	p.value
Control 0 - Ethanol 6	0.9031	0.749	44	1.206	0.7477
Control 0 - Ethanol 10	1.5604	0.769	44	2.029	0.2696
Control 0 - QMP 6	-0.0539	0.749	44	-0.072	1.0000
Control 0 - QMP 10	-0.1871	0.749	44	-0.250	0.9991
Ethanol 6 - Ethanol 10	0.6573	0.769	44	0.855	0.9117
Ethanol 6 - QMP 6	-0.9570	0.749	44	-1.278	0.7056
Ethanol 6 - QMP 10	-1.0902	0.749	44	-1.456	0.5956
Ethanol 10 - QMP 6	-1.6143	0.769	44	-2.099	0.2389
Ethanol 10 - QMP 10	-1.7475	0.769	44	-2.272	0.1734
QMP 6 - QMP 10	-0.1332	0.749	44	-0.178	0.9998

Table 36 – Showing post-hoc pairwise test results from **Figure 3.26B**

contrast	estimate	SE	df	t.ratio	p.value
Control 0 - Ethanol 6	3.629	1.369	29	2.650	0.0871
Control 0 - Ethanol 10	4.475	1.207	29	3.706	0.0073
Control 0 - QMP 6	3.604	1.369	29	2.632	0.0905
Control 0 - QMP 10	3.001	1.186	29	2.531	0.1112
Ethanol 6 - Ethanol 10	0.846	1.207	29	0.701	0.9546
Ethanol 6 - QMP 6	-0.025	1.369	29	-0.018	1.0000
Ethanol 6 - QMP 10	-0.627	1.186	29	-0.529	0.9836
Ethanol 10 - QMP 6	-0.871	1.207	29	-0.722	0.9498
Ethanol 10 - QMP 10	-1.474	0.995	29	-1.482	0.5821
QMP 6 - QMP 10	-0.602	1.186	29	-0.508	0.9859

8.18 Pairwise Test Comparisons for Figure 4.10

Table 37 – Showing post-hoc pairwise test results from **Figure 4.10**

contrast	estimate	SE	df	t.ratio	p.value
Number.of.bees5 - Number.of.bees10	-13.2	58.3	254	-0.226	0.9723
Number.of.bees5 - Number.of.bees15	85.3	54.5	254	1.564	0.2632
Number.of.bees10 - Number.of.bees15	98.5	43.4	254	2.272	0.0617

8.19 Pairwise Test Comparisons for Figure 4.21

Table 38 – Showing post-hoc pairwise test results from **Figure 4.21**

contrast	estimate	SE	df	t.ratio	p.value
Ethanol 5 - Ethanol 10	105.6	83.4	252	1.266	0.8031
Ethanol 5 - Ethanol 15	204.6	81.8	252	2.502	0.1273
Ethanol 5 - QMP 5	186.0	98.3	252	1.893	0.4087
Ethanol 5 - QMP 10	57.0	86.5	252	0.659	0.9862
Ethanol 5 - QMP 15	156.3	77.2	252	2.026	0.3304
Ethanol 10 - Ethanol 15	98.9	63.1	252	1.568	0.6206
Ethanol 10 - QMP 5	80.4	83.3	252	0.965	0.9286
Ethanol 10 - QMP 10	-48.7	72.1	252	-0.675	0.9846
Ethanol 10 - QMP 15	50.7	61.5	252	0.824	0.9629
Ethanol 15 - QMP 5	-18.6	76.4	252	-0.243	0.9999
Ethanol 15 - QMP 10	-147.6	64.0	252	-2.307	0.1952
Ethanol 15 - QMP 15	-48.3	59.0	252	-0.819	0.9639
QMP 5 - QMP 10	-129.1	83.5	252	-1.546	0.6347
QMP 5 - QMP 15	-29.7	80.3	252	-0.370	0.9991
QMP 10 - QMP 15	99.4	65.3	252	1.521	0.6513

8.20 Pairwise Test Comparisons for Figure 4.22

Table 39 – Showing post-hoc pairwise test results from **Figure 4.22**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol 10 - Ethanol 15	-1.0374	0.464	Inf	-2.235	0.2218
Ethanol 10 - Ethanol 5	0.5436	0.688	Inf	0.791	0.9692
Ethanol 10 - QMP 10	0.6580	0.573	Inf	1.149	0.8609
Ethanol 10 - QMP 15	-0.5102	0.463	Inf	-1.101	0.8811
Ethanol 10 - QMP 5	-0.9882	0.595	Inf	-1.662	0.5571
Ethanol 15 - Ethanol 5	1.5810	0.644	Inf	2.454	0.1381
Ethanol 15 - QMP 10	1.6954	0.519	Inf	3.266	0.0139
Ethanol 15 - QMP 15	0.5272	0.393	Inf	1.340	0.7626
Ethanol 15 - QMP 5	0.0492	0.529	Inf	0.093	1.0000
Ethanol 5 - QMP 10	0.1144	0.712	Inf	0.161	1.0000
Ethanol 5 - QMP 15	-1.0538	0.629	Inf	-1.675	0.5485
Ethanol 5 - QMP 5	-1.5318	0.740	Inf	-2.069	0.3037
QMP 10 - QMP 15	-1.1682	0.502	Inf	-2.329	0.1822
QMP 10 - QMP 5	-1.6462	0.635	Inf	-2.594	0.0985
QMP 15 - QMP 5	-0.4780	0.537	Inf	-0.890	0.9490

8.21 Pairwise Test Comparisons for Figure 4.23

Table 40 – Showing post-hoc pairwise test results from **Figure 4.23**

Contrast	estimate	SE	df	t.ratio	p.value
C - D	202.0	63.4	255	3.187	0.0087
C - E	-185.9	82.3	255	-2.259	0.1105
C - F	-108.7	84.1	255	-1.293	0.5685
D - E	-388.0	62.5	255	-6.212	<.0001
D - F	-310.7	64.5	255	-4.821	<.0001
E - F	77.2	83.3	255	0.927	0.7904

8.22 Pairwise Test Comparisons for Figure 4.13

Table 41 – Showing post-hoc pairwise test results from **Figure 4.13**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol CBF - Ethanol Ground	1.756	0.641	Inf	2.739	0.0678
Ethanol CBF - Ethanol Whole	1.482	0.573	Inf	2.585	0.1008
Ethanol CBF - QMP CBF	0.485	0.510	Inf	0.950	0.9332
Ethanol CBF - QMP Ground	0.738	0.516	Inf	1.429	0.7092
Ethanol CBF - QMP Whole	0.122	0.465	Inf	0.263	0.9998
Ethanol Ground - Ethanol Whole	-0.274	0.608	Inf	-0.450	0.9977
Ethanol Ground - QMP CBF	-1.271	0.564	Inf	-2.252	0.2143
Ethanol Ground - QMP Ground	-1.018	0.560	Inf	-1.818	0.4539
Ethanol Ground - QMP Whole	-1.633	0.657	Inf	-2.487	0.1278
Ethanol Whole - QMP CBF	-0.997	0.534	Inf	-1.868	0.4220
Ethanol Whole - QMP Ground	-0.744	0.532	Inf	-1.399	0.7275
Ethanol Whole - QMP Whole	-1.360	0.590	Inf	-2.304	0.1921
QMP CBF - QMP Ground	0.253	0.475	Inf	0.532	0.9949
QMP CBF - QMP Whole	-0.363	0.530	Inf	-0.685	0.9837
QMP Ground - QMP Whole	-0.615	0.535	Inf	-1.150	0.8603

8.23 Pairwise Test Comparisons for Figure 4.15

Table 42 – Showing post-hoc pairwise test results from **Figure 4.15**

contrast	estimate	SE	df	z.ratio	p.value
High - Low	-1.686	0.727	Inf	-2.320	0.0531
High - Med	-0.794	0.672	Inf	-1.181	0.4644
Low - Med	0.892	0.731	Inf	1.221	0.4405

8.24 Pairwise Test Comparisons for Figure 4.25

Table 43 – Showing post-hoc pairwise test results from **Figure 4.25A**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP 0.05	0.4380	0.844	Inf	0.519	0.9986
Ethanol - QMP 0.1	0.0733	0.799	Inf	0.092	1.0000
Ethanol - QMP 0.25	0.1812	0.781	Inf	0.232	1.0000
Ethanol - QMP 0.5	2.8135	1.250	Inf	2.251	0.2682
Ethanol - QMP 1	1.2649	0.835	Inf	1.515	0.7360
Ethanol - QMP 2	0.3213	0.783	Inf	0.411	0.9996
QMP 0.05 - QMP 0.1	-0.3647	0.860	Inf	-0.424	0.9996
QMP 0.05 - QMP 0.25	-0.2568	0.844	Inf	-0.304	0.9999
QMP 0.05 - QMP 0.5	2.3755	1.287	Inf	1.846	0.5168
QMP 0.05 - QMP 1	0.8269	0.893	Inf	0.925	0.9686
QMP 0.05 - QMP 2	-0.1167	0.848	Inf	-0.138	1.0000
QMP 0.1 - QMP 0.25	0.1079	0.766	Inf	0.14	1.0000
QMP 0.1 - QMP 0.5	2.7402	1.229	Inf	2.231	0.2790
QMP 0.1 - QMP 1	1.1916	0.823	Inf	1.447	0.7760
QMP 0.1 - QMP 2	0.2481	0.783	Inf	0.317	0.9999
QMP 0.25 - QMP 0.5	2.6323	1.197	Inf	2.199	0.2960
QMP 0.25 - QMP 1	1.0837	0.787	Inf	1.376	0.8146
QMP 0.25 - QMP 2	0.1402	0.748	Inf	0.187	1.0000
QMP 0.5 - QMP 1	-1.5486	1.229	In	-1.260	0.8700
QMP 0.5 - QMP 2	-2.4921	1.213	Inf	-2.055	0.3799
QMP 1 - QMP 2	-0.9435	0.801	Inf	-1.178	0.9027

Table 44 – Showing post-hoc pairwise test results from **Figure 4.25B**.

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - QMP 0.1	-0.217	0.691	Inf	-0.314	0.9892
Ethanol - QMP 0.5	0.659	0.621	Inf	1.061	0.7133
Ethanol - QMP 1	1.011	0.609	Inf	1.660	0.3449
QMP 0.1 - QMP 0.5	0.877	0.693	Inf	1.265	0.5856
QMP 0.1 - QMP 1	1.229	0.693	Inf	1.773	0.2864
QMP 0.5 - QMP 1	0.352	0.601	Inf	0.585	0.9365

8.25 Pairwise Test Comparisons for Figure 4.17

Table 45 – Showing post-hoc pairwise test results from **Figure 4.17B**

contrast	estimate	SE	df	t.ratio	p.value
Ovary.Score1 - Ovary.Score2	-0.3795	0.564	51	-0.673	0.9069
Ovary.Score1 - Ovary.Score3	-0.2939	0.519	51	-0.566	0.9417
Ovary.Score1 - Ovary.Score4	0.5759	0.463	51	1.244	0.6024
Ovary.Score2 - Ovary.Score3	0.0856	0.514	51	0.167	0.9983
Ovary.Score2 - Ovary.Score4	0.9554	0.452	51	2.115	0.1622
Ovary.Score3 - Ovary.Score4	0.8699	0.366	51	2.376	0.0947

8.26 Pairwise Test Comparisons for Figure 4.18

Table 46 – Showing post-hoc pairwise test results from **Figure 4.18A**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - Pentacosane	-0.0428	0.441	Inf	-0.097	0.9948
Ethanol - QMP	0.2847	0.461	Inf	0.617	0.8107
Pentacosane - QMP	0.3275	0.455	Inf	0.720	0.7516

Table 47 – Showing post-hoc pairwise test results from **Figure 4.18B**

contrast	estimate	SE	df	t.ratio	p.value
Ovary.Score1 - Ovary.Score2	0.201	0.388	104	0.517	0.9547
Ovary.Score1 - Ovary.Score3	-0.321	0.311	104	-1.031	0.7316
Ovary.Score1 - Ovary.Score4	-0.484	0.267	104	-1.814	0.2728
Ovary.Score2 - Ovary.Score3	-0.522	0.388	104	-1.345	0.5367
Ovary.Score2 - Ovary.Score4	-0.685	0.364	104	-1.882	0.2423
Ovary.Score3 - Ovary.Score4	-0.163	0.236	104	-0.691	0.9003

8.27 Pairwise Test Comparisons for Figure 4.19

Table 48 – Showing post-hoc pairwise test results from **Figure 4.19**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - pentacosane	0.789	0.381	Inf	2.070	0.0961
Ethanol - QMP0.25	-0.470	0.272	Inf	-1.726	0.1955
pentacosane - QMP0.25	-1.259	0.359	Inf	-3.511	0.0013

8.28 Pairwise Test Comparisons for Figure 5.3

Table 49 – Showing post-hoc pairwise test results from **Figure 5.3A**

Contrast	estimate	SE	df	z.ratio	p.value
BP - Ethanol	-0.147	0.272	Inf	-0.539	0.8521
BP – QMP	-1.145	0.282	Inf	-4.064	0.0001
Ethanol - QMP	-0.998	0.286	Inf	-3.489	0.0014

Table 50 – Showing post-hoc pairwise test results from **Figure 5.3B**

contrast	estimate	SE	df	z.ratio	p.value
BP – Ethanol	-0.0733	0.270	Inf	-0.271	0.9603
BP - QMP	-0.3039	0.280	Inf	-1.087	0.5221
Ethanol – QMP	-0.2307	0.302	Inf	-0.764	0.7251

Table 51 – Showing post-hoc pairwise test results from **Figure 5.3C**

contrast	estimate	SE	df	z.ratio	p.value
BP - Ethanol	-0.0733	0.270	Inf	-0.271	0.9603
BP - QMP	-0.3039	0.280	Inf	-1.087	0.5221
Ethanol - QMP	-0.2307	0.302	Inf	-0.764	0.7251

Table 52 – Showing post-hoc pairwise test results from **Figure 5.3D**

contrast	estimate	SE	df	z.ratio	p.value
BP – Ethanol	-0.256	0.170	Inf	-1.505	0.2887
BP - QMP	-1.001	0.175	Inf	-5.725	<.0001
Ethanol – QMP	-0.744	0.189	Inf	-3.931	0.0002

Table 53 – Showing post-hoc pairwise test results from **Figure 5.3E**

contrast	estimate	SE	df	z.ratio	p.value
BP - Ethanol	-0.7181	0.307	Inf	-2.341	0.0504
BP - QMP	-0.6504	0.308	Inf	-2.112	0.0875
Ethanol – QMP	0.0677	0.310	Inf	0.218	0.9741

8.29 Pairwise Test Comparisons for Figure 5.4

Table 54 – Showing post-hoc pairwise test results from **Figure 5.4A**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - Ocimene	-0.210	0.226	Inf	-0.928	0.6224
Ethanol - QMP	-0.827	0.253	Inf	-3.268	0.0031
Ocimene - QMP	-0.616	0.245	Inf	-2.521	0.0314

Table 55 – Showing post-hoc pairwise test results from **Figure 5.4B**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - Ocimene	0.809	0.229	Inf	3.532	0.0012
Ethanol - QMP	-0.623	0.276	Inf	-2.253	0.0626
Ocimene - QMP	-1.432	0.261	Inf	-5.478	<.0001

Table 56 – Showing post-hoc pairwise test results from **Figure 5.4C**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - MLNEP	0.194	0.263	Inf	0.738	0.7411
Ethanol - QMP	-0.951	0.275	Inf	-3.459	0.0016
MLNEP - QMP	-1.145	0.279	Inf	-4.100	0.0001

Table 57 – Showing post-hoc pairwise test results from **Figure 5.4D**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - MLNEP	0.249	0.263	Inf	0.946	0.6111
Ethanol - QMP	-0.808	0.274	Inf	-2.952	0.0089
MLNEP - QMP	-1.057	0.274	Inf	-3.865	0.0003

Table 58 – Showing post-hoc pairwise test results from **Figure 5.4E**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - (MLNEP+QMP)	-0.995	0.336	Inf	-2.961	0.0086
Ethanol - QMP	-1.491	0.348	Inf	-4.281	0.0001
(MLNEP+QMP) - QMP	-0.496	0.342	Inf	-1.447	0.3166

8.30 Pairwise Test Comparison for Figure 5.7

Table 59 – Showing post-hoc pairwise test results from **Figure 5.7.**

contrast	estimate	SE	df	t.ratio	p.value
Ethanol - QMP 3.25	1.47	0.625	359	2.351	0.1315
Ethanol - QMP 6.5	2.79	0.629	359	4.442	0.0001
Ethanol - QMP 13	5.99	0.590	359	10.165	<.0001
Ethanol - QMP 26	9.23	0.685	359	13.470	<.0001
QMP 3.25 - QMP 6.5	1.33	0.633	359	2.094	0.2250
QMP 3.25 - QMP 13	4.52	0.594	359	7.617	<.0001
QMP 3.25 - QMP 26	7.77	0.689	359	11.265	<.0001
QMP 6.5 - QMP 13	3.20	0.599	359	5.343	<.0001
QMP 6.5 - QMP 26	6.44	0.693	359	9.288	<.0001
QMP 13 - QMP 26	3.24	0.658	359	4.927	<.0001

8.31 Pairwise Test Comparison for Figure 5.8

Table 60 – Showing post-hoc pairwise test results from **Figure 5.8A**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - EBO111	-0.0257	0.0838	Inf	-0.306	0.9900
Ethanol - EBO1110	0.0518	0.0841	Inf	0.616	0.9270
Ethanol - QMP	1.1849	0.0862	Inf	13.747	<.0001
EBO111 - EBO1110	0.0775	0.0848	Inf	0.914	0.7974
EBO111 - QMP	1.2105	0.0868	Inf	13.944	<.0001
EBO1110 - QMP	1.1330	0.0871	Inf	13.001	<.0001

Table 61 – Showing post-hoc pairwise test results from **Figure 5.8B**

contrast	estimate	SE	df	t.ratio	p.value
Ethanol - BP 61.7	0.3807	0.784	525	0.486	0.9886
Ethanol - BP 617	0.0423	0.788	525	0.054	1.0000
Ethanol - BP 6170	4.4918	0.894	525	5.026	<.0001
Ethanol - QMP	7.5248	0.852	525	8.834	<.0001
BP 61.7 - BP 617	-0.3384	0.767	525	-0.441	0.9921
BP 61.7 - BP 6170	4.1111	0.875	525	4.700	<.0001
BP 61.7 - QMP	7.1441	0.832	525	8.587	<.0001
BP 617 - BP 6170	4.4495	0.879	525	5.063	<.0001
BP 617 - QMP	7.4824	0.836	525	8.948	<.0001
BP 6170 - QMP	3.0330	0.936	525	3.239	0.0111

8.32 Pairwise Test Comparison for Figure 5.9

Table 62 – Showing post-hoc pairwise test results from **Figure 5.9**

contrast	estimate	SE	df	t.ratio	p.value
Ethanol - QMP	-0.01915	0.0269	419	-0.713	0.9534
Ethanol - BP	0.00158	0.0273	419	0.058	1.0000
Ethanol - Commercial BP	-0.03603	0.0298	419	-1.208	0.7465
Ethanol - Pentacosane	-0.11725	0.0338	419	-3.470	0.0052
QMP - BP	0.02073	0.0273	419	0.759	0.9422
QMP - Commercial BP	-0.01687	0.0298	419	-0.566	0.9799
QMP - Pentacosane	-0.09810	0.0338	419	-2.903	0.0317
BP - Commercial BP	-0.03761	0.0302	419	-1.243	0.7259
BP - Pentacosane	-0.11883	0.0342	419	-3.477	0.0051
Commercial BP - Pentacosane	-0.08122	0.0362	419	-2.244	0.1655

8.33 Pairwise Test Comparison for Figure 5.10

Table 63 – Showing post-hoc pairwise test results from **Figure 5.10**

contrast	estimate	SE	df	t.ratio	p.value
Ethanol and Pentane - QMP 6.5	4.821	0.860	279	5.609	<.0001
Ethanol and Pentane - Brood Pheromone	1.184	0.881	279	1.345	0.5351
Ethanol and Pentane - Pentacosane	1.643	0.891	279	1.844	0.2549
QMP 6.5 - Brood Pheromone	-3.637	0.869	279	-4.185	0.0002
QMP 6.5 - Pentacosane	-3.178	0.879	279	-3.614	0.0020
Brood Pheromone - Pentacosane	0.458	0.900	279	0.510	0.9568

8.34 Pairwise Test Comparison for Figure 5.11

Table 64 – Showing post-hoc pairwise test results from **Figure 5.11**

contrast	estimate	SE	df	z.ratio	p.value
Ethanol - 26Qe QMP	1.13652	0.0847	Inf	13.423	<.0001
Ethanol - 10x Quercetin	0.03158	0.0765	Inf	0.413	0.9985
Ethanol - 100x Quercetin	0.09051	0.0785	Inf	1.152	0.8592
Ethanol - (10x Quercetin + 26 Qe QMP)	1.27162	0.0838	Inf	15.179	<.0001
Ethanol - (100x Quercetin + 26 Qe QMP)	1.26530	0.0867	Inf	14.587	<.0001
26Qe QMP - 10x Quercetin	-1.10494	0.0834	Inf	-13.255	<.0001
26Qe QMP - 100x Quercetin	-1.04601	0.0852	Inf	-12.275	<.0001
26Qe QMP - (10x Quercetin + 26 Qe QMP)	0.13510	0.0901	Inf	1.500	0.6643
26Qe QMP - (100x Quercetin + 26 Qe QMP)	0.12877	0.0928	Inf	1.387	0.7349
10x Quercetin - 100x Quercetin	0.05894	0.0771	Inf	0.764	0.9735
10x Quercetin - (10x Quercetin + 26 Qe QMP)	1.24004	0.0825	Inf	15.039	<.0001
10x Quercetin - (100x Quercetin + 26 Qe QMP)	1.23372	0.0855	Inf	14.435	<.0001
100x Quercetin - (10x Quercetin + 26 Qe QMP)	1.18111	0.0843	Inf	14.005	<.0001
100x Quercetin - (100x Quercetin + 26 Qe QMP)	1.17478	0.0873	Inf	13.460	<.0001
(10x Quercetin + 26 Qe QMP) - (100x Quercetin +26 Qe QMP)	-0.00632	0.0920	Inf	-0.069	1.0000

8.35 Pairwise Test Comparisons for Figure 5.12

Table 65 – Showing post-hoc pairwise test results from **Figure 5.12A**

contrast	estimate	SE	df	z.ratio	p.value
(QMP- Quercetin-) - (QMP- Quercetin+)	-0.907	0.664	Inf	-1.365	0.5213
(QMP- Quercetin-) - (QMP+ Quercetin-)	-0.254	0.579	Inf	-0.438	0.9719
(QMP- Quercetin-) - (QMP+ Quercetin+)	-0.640	0.573	Inf	-1.116	0.6794
(QMP- Quercetin+) - (QMP+ Quercetin-)	0.653	0.660	Inf	0.990	0.7551
(QMP- Quercetin+) - (QMP+ Quercetin+)	0.267	0.654	Inf	0.408	0.9771
(QMP+ Quercetin-) - (QMP+ Quercetin+)	-0.386	0.568	Inf	-0.680	0.9047

Table 66 – Showing post-hoc pairwise test results from **Figure 5.12B**

contrast	estimate	SE	df	z.ratio	p.value
DMSOEthanol - DMSOQMP	0.6668	0.157	Inf	4.257	0.0001
DMSOEthanol - QuertEthanol	0.5608	0.150	Inf	3.733	0.0011
DMSOEthanol - QuertQMP	0.6066	0.151	Inf	4.017	0.0003
DMSOQMP - QuertEthanol	-0.1060	0.173	Inf	-0.614	0.9276
DMSOQMP - QuertQMP	-0.0602	0.173	Inf	-0.348	0.9856
QuertEthanol - QuertQMP	0.0458	0.167	Inf	0.274	0.9928

8.36 Pairwise Test Comparisons for Figure 6.3

Table 67 – Showing post-hoc pairwise test results from **Figure 6.3**

contrast	estimate	SE	df	t.ratio	p.value
Ethanol Day 5 - QMP Day 7	7.68	0.654	366	11.749	<.0001
Ethanol Day 5 - Ethanol Day 7	-1.70	0.633	366	-2.679	0.0210
QMP Day 7 - Ethanol Day 7	-9.38	0.531	366	-17.676	<.0001

Chapter 9 - References

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