Biogenic amines as mediators of queen mandibular pheromone's control of worker reproduction in the honey bee, *Apis mellifera*

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The occurrence of reproductive and non-reproductive castes is one of the major hallmarks of eusociality. Constraints on worker reproduction negate conflict over queen-worker reproduction, playing an integral role in the maintenance of colony harmony. The primary aim of this thesis was to improve our understanding of one of the major mechanisms maintaining colony cohesion in honey bees: the inhibition of worker reproduction by QMP. A body of circumstantial evidence already implicated biogenic amines as mediators of QMP's command over the behaviour and ovarian physiology of workers. This thesis aimed to bring further clarity to the roles played by biogenic amines in regulating reproductive constraint, finding evidence that QMP's ability to modulate dopamine and octopamine is likely a pathway through which sterility in workers is induced.

Considering the accumulation of evidence that QMP 'chemically castrates' workers by manipulating fundamental reproductive pathways, the question as to how queens evade the repressive effects of their own pheromone was also addressed. The hypothesis that discrepancies in QMP exposure levels between queens and workers maintains caste-specific differences in QMP's inhibition of reproduction was empirically tested, and recommendations for further study are suggested.

Finally, the importance of QMP to colony function was considered within the context of an applied issue: pesticide-driven declines of honey bee populations. Despite QMP being central to honey bee biology, its interactive effects with commonly used pesticides has been under-researched. I identify a number of synergistic effects between QMP and the neonicotinoid pesticide imidacloprid on workers which may have negative effects for colony health and productivity. Given the demonstrated breadth of QMP's effects on the neuroendocrine system, reproductive physiology, and behaviour of workers, a case is made for the inclusion of QMP in future assessments of pesticide lethal and sub-lethal effects on honey bees.

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List of Abbreviations

20E – 20-Hydroxyecdysone

5-HT – serotonin

9-HDA - (E)-9-hydroxydec-2-onoic acid

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

ACh – Acetylcholine

AChE - Acetylcholine esterase

CA – Corporus allatum

CBF – complete bee food

CI – confidence interval

CLMM – cumulative link mixed effects model

CNS – central nervous system

CPH – Cox proportional hazards

DA – dopamine

ETH – ecdysis-triggering hormone

GLMM – generalized linear mixed effects model

HOB - methyl p-hydroxybenzoate

HPLC-FLD – high performance liquid chromatography with fluorescent detection

HR – hazard ratio

HVA - 4-hydroxy-3-methoxyphenylethanol

IMI – imidacloprid

JH – juvenile hormone

LMM – linear mixed effects model

nAChR – nicotinic acetylcholine receptor

NE – norepinephrine

OA – octopamine

PBS – phosphate buffered saline

ppb – parts per billion

QE – queen equivalent

QE/µL - queen equivalents per microliter

QL – queenless

QMP – Queen mandibular pheromone

QR – queenright

RH – relative humidity

RPM – revolutions per minute

TA – tyramine

Vg – vitellogenin

YP – Yolk protein

Chapter 1

General Introduction

1.1 Eusociality

1.1.1 The success of social living

One of the major transitions in evolution was the shift from solitary individuals to eusocial colonies (Szathmáry & Smith, 1995). Characterised by the occurrence of reproductive and non-reproductive castes within a society (Wilson 1971), eusociality is a phenomenon that has confounded biologists for centuries. Charles Darwin himself struggled to reconcile the evolution of an effectively sterile worker caste with his theory of evolution by natural selection, famously citing eusociality as his 'one special difficulty, which at first appeared to me insuperable, and actually fatal to my whole theory' (Darwin, 1859).

Although various definitions of eusociality exist, the most widely accepted involves three key features that define eusociality: co-operative brood care, reproductive division of labour, and the overlap of generations living together (Wilson, 1975). The success of this life history strategy is demonstrated in the extent to which eusocial insects dominate the insect fauna of the ecosystems in which they live (Wilson, 1990). Most strikingly, although less than 2% of insect species are eusocial, they constitute an estimated 50% of the biomass in some ecosystems (Wilson, 2008).

Eusociality has evolved numerous times in insects, in further testament to the success of this life history strategy (Wilson & Hölldobler, 2005). Eusociality is most common within the Hymenoptera, within which this high level of social organisation has seen up to nine independent origins (Hughes *et al*., 2008). However, it has also arisen in several other invertebrate orders, including Isoptera (Wilson, 1975), Hemiptera (Stern, 1994), Coleoptera (Smith *et al*., 2009), Thysanoptera (Gadagkar, 1993), and a sponge-dwelling shrimp (Duffy, 1973). Thanks to naked mole-rats and damaraland mole-rats, there are even examples of eusociality in mammals (Burland *et al*., 2002; Jarvis, 1981). With this,

it is unsurprising that eusociality has drawn substantial scientific interest, amassing decades of research attention.

1.1.2 Inclusive fitness theory

The facet of eusocial biology of particular interest to many researchers is its evolution. In fact, the question of how an effectively sterile caste could evolve under natural selection has been a source of much debate. Two opposing theories outline how eusociality may evolve: Inclusive Fitness (Hamilton, 1964b, 1964a) and Group Selection (Nowak *et al*., 2010; Wilson & Wilson, 2007).

Group selection theory posits that eusociality evolved through positive selection on groups of individuals due to the advantages of living together co-operatively (Nowak *et al*., 2010; Wilson & Wilson, 2007). However, the central idea that selection could act at the level of a group has been widely criticized, as such a system would be highly vulnerable to cheating by individuals. On the other hand, Hamilton's theory of inclusive fitness (Hamilton, 1964a, 1964b), also known as "kin selection" (Maynard Smith, 1964), was the first proposed theory and remains the most widely accepted today (West & Gardner, 2013). It is rooted in the idea that an individual can accrue indirect fitness benefits from helping to rear the offspring of a close relation, as this aids the transfer of their genes to the next generation (Hamilton, 1964a, 1964b). This provides a general explanation for the reproductive division of labour that defines eusocial insects, as the intermediate levels of relatedness typically found in insect societies increase the benefits of altruism. With this, workers theoretically benefit from investing in the reproduction of the queen through co-operative brood care, as opposed to investing in their own direct fitness by attempting to reproduce themselves (Hamilton, 1964a, 1964b).

1.1.3 Conflict over reproduction

The inclusive fitness theory provides us a useful framework for understanding the ultimate mechanisms behind the evolution of eusociality, specifically the reproductive division of labour. However, extant eusocial insect societies are still subject to internal conflicts over reproduction (Ratnieks & Reeve, 1992). For instance, workers in a range of

eusocial species may attempt to increase their direct fitness by laying eggs (Bourke, 1988; Hammond & Keller, 2004; Wenseleers *et al*., 2004), attempting to develop into queens (Bourke & Ratnieks, 1999; Wenseleers *et al*., 2003), or even overthrowing the existing queen in species without morphologically distinct queens and workers (i.e. ponerine ants; Monnin *et al*., 2002; Monnin & Ratnieks, 2001). Such conflicts have driven the evolution of numerous mechanisms that constrain the reproductive capabilities of workers relative to queens, known as reproductive constraints (Khila & Abouheif, 2008, 2010). By negating conflict over queen-worker reproduction, reproductive constraints are integral to the maintenance of social harmony within the colony (Ratnieks *et al*., 2006). An appreciation of the mechanisms of reproductive constraint in social insects is thus fundamental to our understanding of the evolution of eusociality.

1.2 Reproductive constraints

Reproductive constraints constitute any mechanism that reduces the ability of workers to reproduce relative to the queen (Khila & Abouheif, 2010). This encompasses a diverse range of processes, from behavioural – such as the policing of worker-laid eggs (e.g. Wenseleers & Ratnieks, 2006) – to developmental and physiological constraints. Khila & Abouheif (2010) further classified the developmental reproductive constraints of ant species into five sub-categories, termed RC1-RC5 (Table 1.1). Although these sub-categories describe the developmental constraints present in ants, they are widely applicable to a variety of eusocial species.

Reproductive constraint	**Mechanism**
RC1	Mis-localisation of mRNA
RC2	Quantitative constraint on ovary activity
RC3	Loss of spermatheca
RC4	Reduction of ovariole number
RC5	Complete loss of reproductive organs

Table 1.1 Reproductive constraints. The five classifications of developmental reproductive constraints in ants described by Khila and Abouheif (2010). Reproductive constraints may be reversible (RC1-RC2) or irreversible (RC3-5).

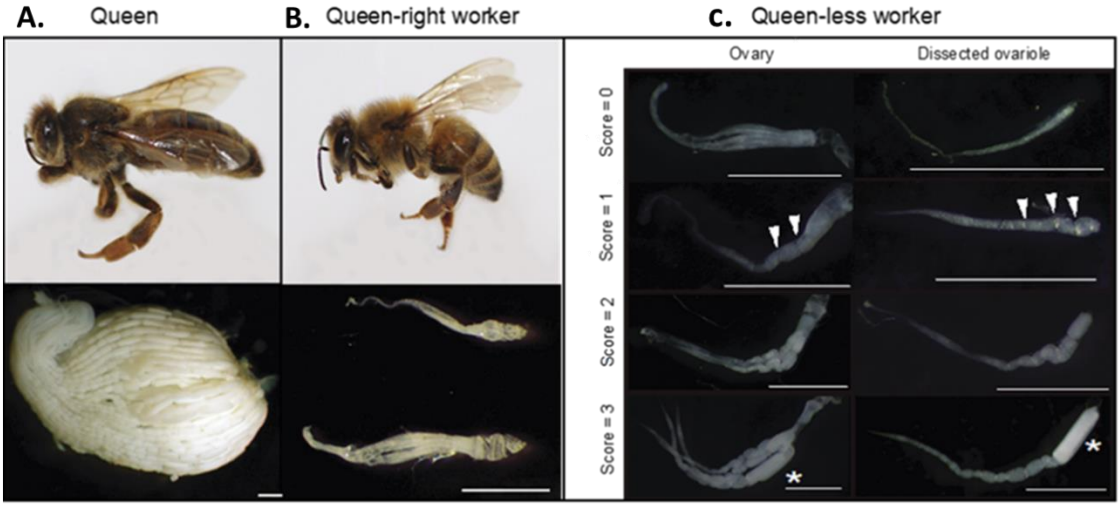

Figure 1.1 Differences in honey bee ovary morphology between castes Differences in honey bee *Apis mellifera* ovary physiology between **A.** Queens, **B.** Queen-right workers (i.e. workers from a hive headed by a queen), and **C.** Queen-less workers (i.e. workers from a hive lacking a queen). Workers develop considerably fewer ovarioles than queens (where ovarioles are individual strings of developing oocytes; **C.** pictures full ovaries and the dissected individual ovarioles), and also lack a spermatheca (not pictured). In the absence of a queen, workers may activate their ovaries and produce male eggs. **C.** shows the four distinct stages of ovary activity, ranging from inactive (0) to possessing mature oocytes (3). Mature oocytes are denoted by asterisks, while arrows mark signs of differentiation (a detailed explanation of each stage is found in Chapter 2). Scale bars indicate 1 mm. Figure provided by Dr Elizabeth J. Duncan and used with permission.

1.2.1 Irreversible constraints

Constraints on worker physiology reduce their ability to reproduce in ways which are both reversible (i.e. RC1-RC2) and irreversible (i.e. RC3-RC5). The most extreme constraint, the complete loss of reproductive organs (RC5), is the only constraint that completely eliminates worker reproduction and is comparatively rare; It is found in only nine out of 283 ant genera (Khila & Abouheif, 2010). Other irreversible developmental constraints merely quantitatively or qualitatively lower the reproductive output of workers. For example, while the loss of the spermatheca (sperm storage organ) prevents hymenopteran workers from laying fertilized female eggs (RC3), they are still able to produce unfertilized eggs destined to become males due to their haplodiploid sex determination system (e.g. honey bees: Snodgrass 1956; some ant species: Khila & Abouheif, 2010). Reductions in ovariole numbers (ovarioles are strings of developing

oocytes; Figure 1.1; RC4) are another irreversible developmental constraint which generally accompanies the loss of the spermatheca, arising during embryonic development in ants (Khila & Abouheif, 2010) and larval development in honey bees (Reginato & Cruz-Landim, 2003; Schmidt, Capella & Hartfelder, 2002). Ovariole loss severely reduces, but does not completely eliminate, the reproductive potential of workers relative to queens. In honey bees for instance, the feeding of royal jelly during the larval stage triggers the development of considerably larger ovaries in queens relative to workers, with queen ovaries consisting of 120-200 ovarioles while workers develop as few as 2-12 (Figure 1.1; Snodgrass 1956; Linksvayer *et al*., 2011).

1.2.2 Reversible constraints

In contrast to these irreversible reductions to worker fecundity, some reproductive constraints confer an element of plasticity in adulthood based on environmental signals. For instance, quantitative constraints on ovary activity (RC2) are not an ultimate constraint, and in certain social conditions, workers have the capacity to increase their ovary activity and begin laying their own eggs (Khila & Abouheif, 2010). This is likely an adaptive developmental mechanism, allowing workers to regulate their reproductive physiology such that they can increase their fitness either indirectly (i.e. through the indirect fitness benefits of engaging in co-operative brood care) or directly (i.e. by activating their ovaries and reproducing themselves) depending on social cues.

The most common situation in which workers may favour their own reproduction is in the absence of a queen. For example, queenless (QL) workers (i.e. workers from a colony in which the queen or dominant reproductive has been lost) have been seen to activate their ovaries in many eusocial species, including the ponerine ant *Harpegnathos saltator* (Peeters *et al*., 2000; Peeters & Hölldobler, 1995), the bumble bee *Bombus terrestris* (i.e. van Oystaeyen *et al*., 2014), and the honey bee (Hoover *et al*., 2003) to name just a few. To this end, queen pheromones play a key role in the mediation of RC2 by signalling the queen's presence and/or suppressing worker reproduction (le Conte & Hefetz, 2008). As with other reproductive constraints, the inhibition of worker reproduction by queen

pheromones is an integral mechanism for the maintenance of colony harmony. Understanding how these pheromones have arisen, and the mechanisms by which they inhibit worker reproduction, is therefore central to the evolution of eusociality.

1.3 Queen pheromones

1.3.1 Ancestral Hymenopteran queen pheromones

Across the eusocial Hymenoptera, queens produce pheromones which signal their presence to workers. These queen pheromones show a remarkable degree of conservation across at least three independent origins of eusociality (Figure 1.2). Queens of three species of ants (Holman *et al*., 2010, 2013; van Oystaeyen *et al*., 2014), the bumble bee *Bombus terrestris* (Holman, 2014; van Oystaeyen *et al*., 2014) and the common wasp *Vespula vulgaris* (van Oystaeyen *et al*., 2014) all produce sterility-inducing

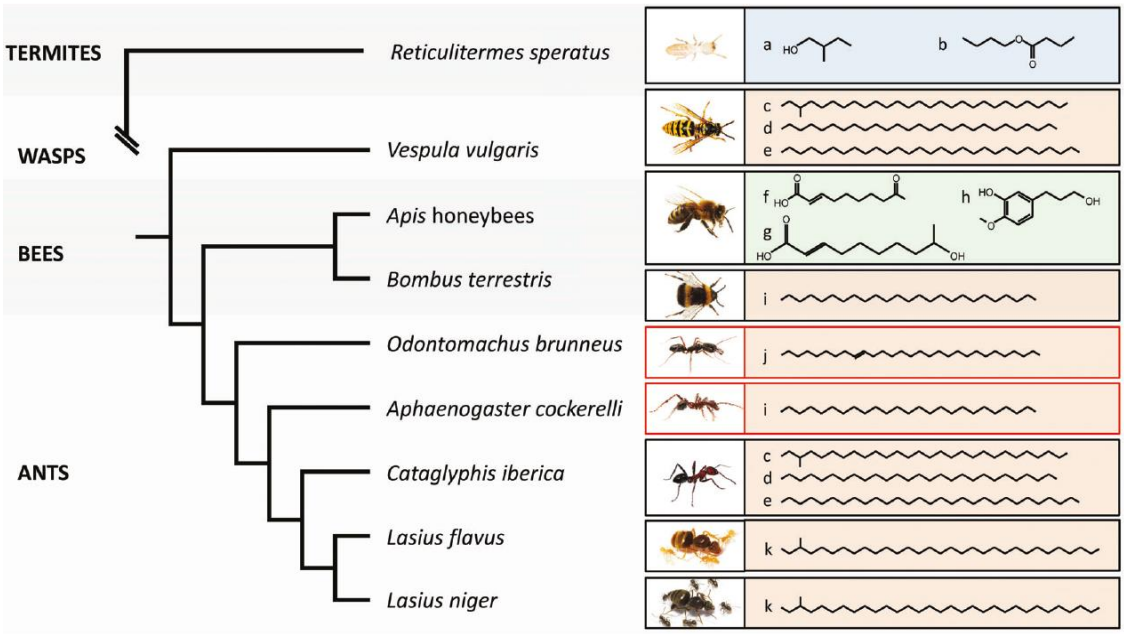

Figure 1.2 Evolutionary history of queen pheromones in eusocial insects demonstrating the structural difference of honey bee queen pheromone (QMP) and termite queen pheromones with those used in other species. Alternately shaded areas represent independent origins of eusociality. Non-volatile cuticular hydrocarbons (peach) are used as conserved queen pheromones in ants, bumble bees and vespine wasps (Holman et al., 2010, 2013; van Oystaeyen et al., 2014). Honey bees (light green) and termites (light blue) use other volatile compounds including keto acids, esters, and alcohols (Hoover et al., 2003; Matsuura et al., 2010; K. N. Slessor et al., 2005). Figure from Oi et al., (2015), used with author's permission.

queen signals in their cuticle consisting of non-volatile long-chain linear and methyl-branched saturated hydrocarbons (Figure 1.2). The commonality of these non-volatile hydrocarbon components across diverse taxa indicates that they were likely derived from a signalling system already present in their solitary ancestors. Interestingly, similar long-chain hydrocarbons serve as signals of fertility in solitary species, presumably for the purpose of mate attraction (Oi *et al*., 2015). Evidence suggests that the production of long-chain hydrocarbons is a direct by-product of ovary development, implicating these signals as "honest" indicators of fertility (i.e. cannot be cheated). In the house fly, for instance, production of the cuticular hydrocarbon sex pheromone (Z)-9-tricosene is directly triggered by ovary activity (Blomquist & Vogt, 2003). Phylogenetic analyses show that queen signals have a high degree of conservation with these fertility signals (Holman *et al*., 2013; van Oystaeyen *et al*., 2014), indicating that the majority of eusocial queen pheromones likely have origins as honest signals of fecundity.

1.3.2 Queen mandibular pheromone

One of the earliest discovered and best studied queen pheromones is the honey bee's queen mandibular pheromone (QMP). As the name suggests, QMP is produced not in the queen's cuticle, but in her mandibular glands (Slessor *et al*., 1988). QMP's principal components differ considerably from the assumed ancestral non-volatile hydrocarbons described above, instead containing a blend of volatile carboxylic acids: (E)-9-oxodec-2-enoic acid (9-ODA) and two enantiomers of (E)-9-hydroxydec-2-onoic acid (9-HDA); two aromatics: metyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA); and other minor compounds (Figure 1.3; Keeling *et al*., 2003; Slessor *et al*., 1988). This blend of five semiochemicals inhibits the activity of worker ovaries (Hoover *et al*., 2003). QMP serves further roles in addition to the induction of sterility in workers, including attracting drones during mating flights (Winston & Slessor, 1998), the inhibition of queen rearing (Winston *et al*., 1990), and the attraction of workers to the queen, known as retinue behaviour (Slessor *et al*., 1988). As such, QMP is considerably more complex than the cuticular-hydrocarbon based pheromones that predominate the

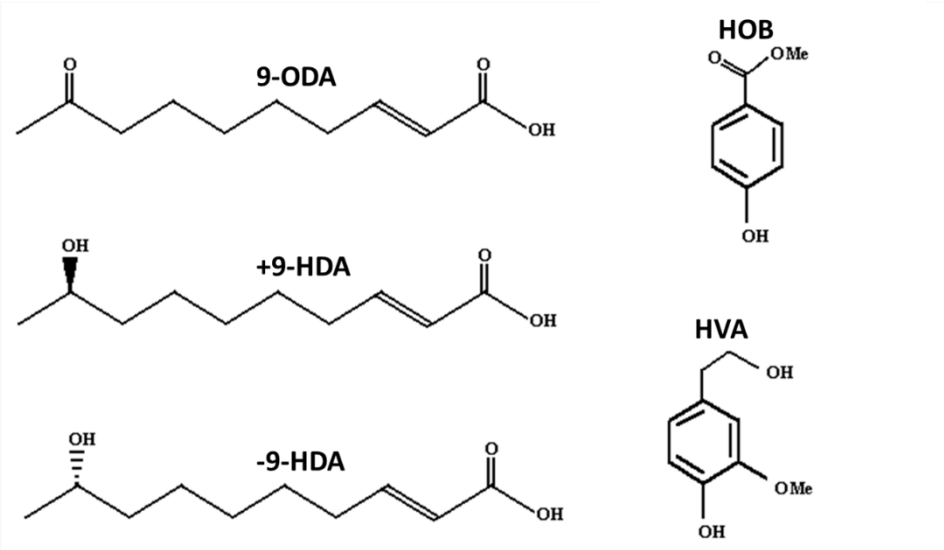

Figure 1.3 Chemical structures of the five major QMP components: 9-ODA (E-9-oxodec-2-enoic acid), both enantiomers of 9-HDA (E-9-hydroxydec-2-onoic acid), HOB (metyl p-hydroxybenzoate) and HVA (4-hydroxy-3-methoxyphenylethanol)

rest of the eusocial Hymenoptera, in terms of both its chemical complexity and its wide range of functions.

However, QMP is unlikely the only signal used by honey bee queens that effects worker reproduction, as queens from which the mandibular gland has been removed are still able to inhibit ovary activity (Maisonnasse, Alaux, *et al*., 2010). Recent findings suggest cuticular hydrocarbons may also serve as repressive queen signals in honey bees, as tergal gland esters present on the queen's dorsal cuticle have been shown to inhibit the reproduction of workers in addition to QMP (Princen *et al*., 2019). The brood pheromones ethyl palminate, methyl linoleate and the brood volatile E-β-ocimene are also inhibitors of worker reproduction (Maisonnasse, Lenoir, *et al*., 2010; Mohammedi *et al*., 1998; Traynor *et al*., 2014), and likely encourage workers to care for the brood. There is now understood to be multiple levels of redundancy in the signals that repress worker reproduction in honey bees (Princen *et al*., 2019). How this species came to possess such a highly derived and complex pheromone system makes them an interesting model for understanding the evolution of queen pheromones.

1.4 The Honest signal vs Queen control hypotheses

1.4.1 Honest signal hypothesis

Regarding the evolution of queen pheromones, there is debate as to whether these pheromones function as honest signals for the presence of a fecund queen (honest signal hypothesis; Keller & Nonacs, 1993; Monnin, 2006; Seeley, 1985), or as manipulative compounds that chemically enforce sterilization upon workers against their reproductive interests (queen control hypothesis; Fletcher & Ross, 1985; Strauss *et al*., 2008; Hefetz 2004; Hölldobler & Wilson 1990). Under the honest signal hypothesis, it is thought that workers under the presence of a fecund queen benefit more from rearing the queen's brood (i.e. their siblings) than attempting to reproduce themselves (Keller & Nonacs, 1993). As such, queen pheromones should be selected for their ability to reliably or 'honestly' signal the queen's fecundity (Oi *et al*., 2015). The prevalence of a conserved class of fertility-linked cuticular hydrocarbons as queen pheromones lends considerable support to the honest signal hypothesis (Holman *et al*., 2010, 2013; van Oystaeyen *et al*., 2014). In addition, even workers commonly begin producing queen-like pheromones as they activate their ovaries, despite this making them more vulnerable to attack by policing workers (i.e. in social wasps; Smith *et al*., 2009; van Zweden *et al*., 2014), implicating these signals as uncheatable outcomes of fertility (Holman, 2012; Peso *et al*., 2015).

Although QMP's chemical composition is considerably removed from the fertility-linked cuticular hydrocarbons used as queen pheromones in other species, evidence points to it also serving as an honest indicator of fecundity. The production of QMP is tightly linked with the queen's reproductive state (Kocher *et al*., 2009; see Table 1.2). For instance, the quantity and chemical composition of QMP produced differs between virgin and mated queens (Plettner *et al*., 1997), between laying vs non-laying mated queens (Kocher *et al*., 2008), with the number of drones she has mated with (Richard *et al*., 2007), and with semen insemination volume (Niño *et al*., 2012). A function of QMP as an

QMP component	Functions	Abundance in virgin vs mated queens
(E)-9-oxodec-2-enoic acid (ODA)	Complete inhibition of worker reproduction (Princen *et al.,* 2019; Tan *et al.,* 2015; Butler & Fairey, 1963) Weak retinue response (Complete QMP blend required for full retinue response; Slessor *et al.,* 1988) Does **not** inhibit queen rearing (Butler & Fairey, 1963)	Quantity produced increases from young virgin to 1-year old mated queens (Plettner *et al.,* 1997) Ratio of ODA to 10-HDA increases with queen age and mating status (Plettner *et al.,* 1997)
(E)-9-hydroxydec-2-onoic acid (9-HDA)	Complete inhibition of worker reproduction (Princen *et al.,* 2019; Tan *et al.,* 2015) Weak retinue response (Complete QMP blend required for full retinue response; Slessor *et al.,* 1988)	Quantity produced increases from young virgin to 1-year old mated queens (Plettner *et al.,* 1997) Higher ratio of 9-HDA to 10-HDA indicative of "higher quality" queens (Rangel *et al.,* 2016)
Methyl p-hydroxybenzoate (HOB)	Partial inhibition of worker reproduction (Princen *et al.,* 2019) Weak retinue response (Complete QMP blend required for full retinue response; Slessor *et al.,* 1988)	Quantity produced increases from young virgin to 1-year old mated queens (Plettner *et al.,* 1997)
4-hydroxy-3-methoxyphenylethanol (HVA)	Partial inhibition of worker reproduction (Princen *et al.,* 2019) Weak retinue response (Complete QMP blend required for full retinue response; Slessor *et al.,* 1988)	Produced only by 1-year old mated but not young virgin queens (Plettner *et al.,* 1997)

Table 1.2 Principal QMP components and their functions. A summary of what is currently known about the functions of each of the principal QMP components in isolation and how their abundance differs between virgin and mated queens

honest indicator of fecundity even in this highly derived pheromone provides additional support for the queen signal hypothesis.

1.4.2 Queen control hypothesis – is QMP a suppressive agent?

The queen control hypothesis, on the other hand, suggests that queen pheromones act as suppressive agents and chemically sterilize workers against their best interests (Fletcher & Ross, 1985; Strauss *et al*., 2008; Hefetz 2004; Hölldobler & Wilson 1990). Under this model, it is predicted that queen pheromones should evolve rapidly owing to an evolutionary 'arms race' between queens a workers, whereby workers are continually selected to evade repression and queens are under pressure to evolve novel repressive agents (Heinze & D'Ettorre, 2009). The remarkably conserved class of cuticular hydrocarbon-based queen pheromones do not support this (van Oystaeyen *et al*., 2014). However, the complexity of honey bee QMP (Slessor *et al*., 1990) and the functional redundancy of its components (Princen *et al*., 2019) is in line with the predicted 'arms race' of the queen control hypothesis. Could the highly derived QMP have taken on a novel function as a suppressive agent?

There is evidence pointing towards QMP chemically suppressing reproduction. Most convincing is the fact that QMP has been found to repress reproduction across a broad phylogeny of species. For instance, honey bee QMP represses oogenesis in virgin *Drosophila melanogaster* females (Camiletti *et al*., 2013), despite these species being ~340 million years diverged (Misof *et al*., 2014). Reproduction is also repressed by QMP in a species of ant (Carlisle & Butler, 1956), a house fly (Nayar, 1963), a termite (Hrdy *et al*., 1960), and even a prawn (Carlisle & Butler, 1956). There is clearly no adaptive benefit in being responsive to honey bee QMP in these species, as is predicted for worker responses under the queen signal hypothesis. Instead, it seems likely that QMP may have evolved to chemically repress worker reproduction by targeting pathways involved in regulating reproduction already present in solitary ancestors. This modulation of reproductive pathways appears to be a feature unique to QMP, as ancestral

hymenopteran queen pheromones do not share these broad phylogenetic repressive effects (Lovegrove *et al*., 2019). Despite QMP being one of the most highly studied queen pheromones, we still lack a complete understanding of the specific molecular pathways involved in its repression of ovary activity. Improving our understanding of the mechanisms underlying QMP's suppression of reproduction is therefore key to addressing whether this pheromone serves as an agent of sterilization, as predicted by the queen control hypothesis, in addition to being an honest signal of fecundity.

1.5 How does QMP inhibit reproduction?

1.5.1 Ovarian response to QMP – Notch signalling

Although the complete molecular signalling pathway of QMP's induction of sterility has not yet been fully elucidated, we have a good understanding of the ovarian response to QMP. In the presence of QMP, ovary development is understood to be inhibited by active Notch signalling, which acts in the germarium to block oogenesis (Figure 1.4; Duncan *et al*., 2016). Oocytes that do develop are then possibly destroyed via programmed cell death (Ronai *et al*., 2015). Active Notch signalling is triggered by the binding of the ligands Delta and Serrate to the Notch receptor, which is cleaved by the γ-secretase complex. This allows the translocation of the Notch intracellular domain (NICD) to the nucleus, leading to the recruitment of histone acetyltransferases (HATs) which enable the transcription of the enhancer of split complex E(spl)-C and other genes (Bray, 2006). This is an ancient and pleiotropic signalling pathway, with functions in the development of tissues and organs in a diverse range of species (Guruharsha *et al*., 2012).

Importantly, Notch signalling has been demonstrated to be responsive to environmental cues (Hsu & Drummond-Barbosa, 2011). For instance, in *D. melanogaster,* it is involved in regulating reproduction in response to nutrient availability (Bonfini *et al*., 2015). Duncan *et al*., (2016)'s finding that QMP activates Notch signalling to inhibit oogenesis in workers indicates that this ancient, environmentally responsive signalling pathway has been co-opted into regulating reproductive constraint in honey bees. However, there

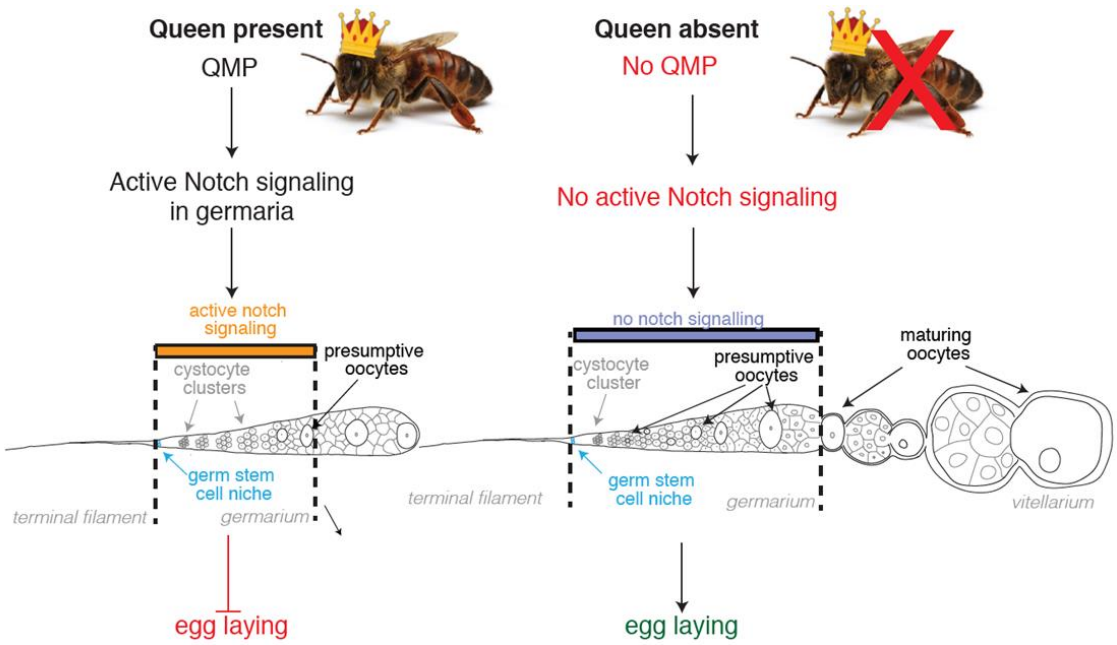

Figure 1.4 Schematic of Notch signalling's inhibition of oogenesis in response to QMP. In the presence of QMP, Notch signalling is active in the germaria (region of the ovaries where germ cells are specified) and egg production is blocked **(Left)**. In the absence of QMP, there is no active Notch signalling in the germaria, allowing presumptive oocytes to mature into fully developed eggs **(Right)**. Figure modified from Duncan et al., (2016), used with permission.

are still gaps in our knowledge as to how QMP triggers the activation of Notch signalling. In particular, we lack understanding of the mechanism linking the detection of QMP by workers with the transmission of this signal to the ovary. To understand how QMP may exert its effects on ovary physiology, we must look to the neuroendocrine signalling pathways governing reproduction in honey bees.

1.5.2 Neuroendocrine control of reproduction

In all animals, the neuroendocrine system links the regulation of reproduction with environmental cues. In insects, the core neuroendocrine system incorporates nutrient sensing (e.g. insulin signalling), hormones (e.g. juvenile hormone and 20-hydroxyecdysone) and biogenic amines (e.g. dopamine and octopamine) (Figure 1.5; Reviewed by Knapp *et al.,* 2022). Much of our understanding of how these neuroendocrine signalling channels regulate reproduction and oogenesis comes from research carried out in D. melanogaster. In D. melanogaster, vitellogenesis (i.e. the

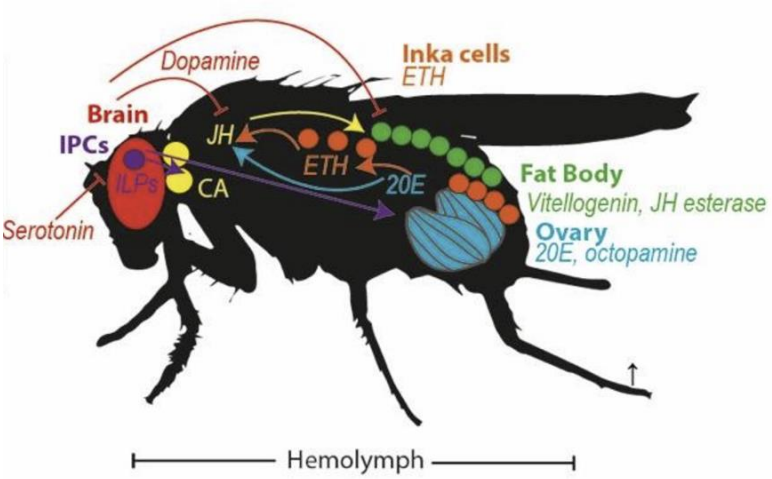

Figure 1.5 Major neuroendocrine signalling pathways involved in regulating reproduction in response to environmental cues in *Drosophila melanogaster*. Figure from Knapp, Norman *et al*., (2022), In press.

accumulation of yolk into developing oocytes) is regulated by juvenile hormone (JH) and the insect steroid hormones ecdysteroids. These hormones act together to stimulate yolk protein (YP) synthesis in the fat body and uptake of YP by the ovary (Jowett & Postlethwait, 1980; Postlethwait & Handler, 1979). The synthesis of JH occurs in the corporus allatum (CA), and is triggered by stimulation of the endocrine Inka cells by 20-hydroxyecysone (20E) to express and secrete ecdysis-triggering hormone (ETH). This regulatory pathway is responsive to environmental stimuli. For instance, vitellogenesis, ovulation and oviposition are triggered post-mating by the seminal substance sex peptide (SP), which acts via neuropeptide allatostatin-C (AstC)-producing neurons which gate the biosynthesis of JH (Zhang *et al*., 2022).

While the regulation of oogenesis is well-understood in D. melanogaster, a shortage of empirical evidence from other species makes it difficult to understand how representative the reproductive neuroendocrine network of this model organism is to other insect species. For instance, an important distinction between neuroendocondrine regulation of oogenesis in honey bees vs D. melanogaster is the loss of JH's gonadotropic function. In honey bees, JH titres have been decoupled from the production of vitellogenin, and there is no evidence to suggest a role of JH in the regulation of reproduction (Rodrigues & Flatt, 2016). How the neuroendocrine network has been

rewired to allow JH to lose this function in honey bees is not completely understood. In particular, it is not clear what may have taken on JH's role as the principal gonadotropin. This leads to interest in alternative signalling molecules involved in regulating reproduction in honey bees, as these would be likely targets for QMP to induce sterility in workers.

1.5.3 Biogenic amines

Among their diverse suite of functions, biogenic amines such as dopamine, serotonin, octopamine and tyramine are also involved in mediating reproduction in insects. These compounds coordinate a range of behavioural and physiological processes though their functions as neurotransmitters, neuromodulators and circulating neurohormones (reviewed by Sasaki & Harano, 2010). Many biogenic amines have overlapping properties in insects, functioning as neurotransmitters in localised interneuronal signalling and as neurohormones when released into the hemolymph and transported to target peripheral tissues via the circulatory system (Blenau & Baumann, 2001). The neural and endocrine functions of these compounds presumably also enable the mediation of insect behaviour and physiology over differing timescales. It is well known that neural connections produce rapid-fire electrochemical signals occurring on the millisecond scale, while the endocrine system functions over a longer time frame. For instance, in *Drosophila virilis,* the comparatively rapid neuroendocrine stress response produces an elevation in dopamine, tyramine and octopamine levels 15 minutes after exposure to a stressor (Hirashima *et al.* 2000).

The general functions of biogenic amines in insects have been well documented. Dopamine has roles both as a slow neurohormone and fast neurotransmitter in insects. In flies, for instance, dopamine neurons co-ordinate processes such as learning and memory, sexual drive, sleep, and hunger (Ichinose et al., 2017). Meanwhile, octopamine and its precursor tyramine are the invertebrate equivalents of vertebrate adrenergic transmitters, acting antagonistically to regulate the "fight or flight" response (Roeder, 2005). In invertebrate peripheral nervous systems, octopamine also regulates flight

muscle activity and energy metabolism, and can modulate peripheral and sense organs such as the fat body, oviduct and hemocytes (Goosey & Candy, 1982; Orchard *et al.* 1982; Adamo *et al.* 1995). In the central nervous system, octopamine is also involved in regulating motivation and initiation, sensory inputs, and maintaining complex behaviours including learning and memory (Farooqui, 2007).

In addition to these general functions, biogenic amines are involved in regulating reproduction in many insect secies primarily via the neuroendocrine system. These biogenic amines indirectly affect reproduction by regulating JH titres in *Drosophila* species (e.g. Gruntenko *et al*., 2005, 2007), but can also directly regulate oogenesis and oviposition (Monastirioti, 2003; White *et al*., 2021). Correlative evidence suggests that biogenic amines may regulate reproduction in a range of social Hymenopterans. In *Polistes* paper wasps, brain levels of dopamine, serotonin and their metabolites are positively correlated with ovarian width in workers with developed ovaries (Sasaki *et al*., 2007). An increase in serotonin and octopamine in the brain is associated with the shift from pre-reproductive to reproductive stages in the facultatively social carpenter bee, *Certina calcarata* (Cook *et al*., 2019). Brain dopamine levels correlate positively with terminal oocyte length in bumble bees (Bloch *et al*., 2000; Sasaki *et al*., 2017) and with ovarian activity in the secondarily queenless (QL) ant *Harpegnathos saltator* (Penick *et al*., 2014) and QL honey bee workers (Sasaki & Nagao, 2001). Manipulative studies are less common, but those that have been carried out also implicate biogenic amines, in particular dopamine, in regulating reproduction. For example, inhibiting dopamine synthesis reduced rates of egg-laying and numbers of chorionated oocytes in the fire ant *Solenopsis invicta* (Boulay *et al*., 2001). Further, dopamine application has been found to enhance ovary activity in a derived QL *Diacamma* ant (Okada *et al*., 2015) and in QL honey bees (Dombroski *et al*., 2003). This implicates biogenic amines as being important for the regulation of reproduction across a broad range of eusocial Hymenopterans.

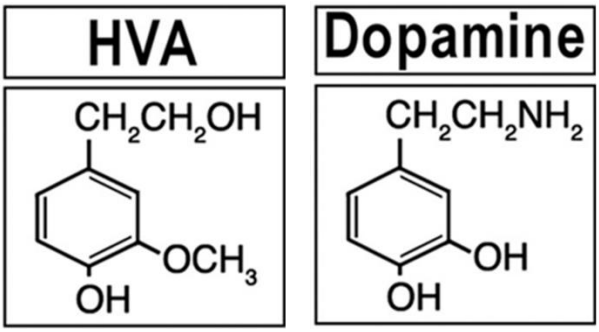

Figure 1.6 One of QMP's core components, HVA, is structurally very similar to dopamine. Figure modified from (Beggs et al., 2007)

Given this large body of evidence linking biogenic amines with ovary development, studies have begun to address whether this may be a target pathway for QMP in its regulation of reproductive constraint in honey bees (Figure 1.7). Circumstantial evidence implicates dopamine in particular as a mediator of QMP's repression of ovary activity. For instance, brain dopamine levels are lower in QR relative to QL workers (Harris & Woodring, 1995) and positively correlate with ovary activity in QL workers (Sasaki & Nagao, 2001). Importantly, there is also evidence that QMP can directly modulate dopamine levels; One of QMP's five principal components HVA has been shown to reduce dopamine titres in the brains of workers (Beggs *et al*., 2007). The exact mechanism by which this occurs is not fully understood. However, HVA shares remarkable structural similarity with dopamine (Figure 1.6) and can selectively bind to the dopamine receptor AmDop3 (Beggs & Mercer, 2009), which may underly its capacity to directly modulate dopamine signalling. HVA's effects on the dopaminergic system have been directly linked with QMP's regulation of aversive learning and memory in young workers (Vergoz *et al*., 2007). However, it is unclear whether these modulations also affect worker ovarian development (Figure 1.7). Considering the suspected role of dopamine as a gonadotropin in a range of social Hymenopterans (i.e. as discussed above), HVA's suppression of dopamine could plausibly elicit sterilizing effects in honey bee workers. This raises the intriguing possibility that as well as being an honest indicator of fecundity (Kocher *et al*., 2009), QMP is capable of inhibiting the reproduction of workers by directly manipulating signalling molecules with likely pre-existing roles in regulating reproduction: biogenic amines. This would add to existing evidence for an

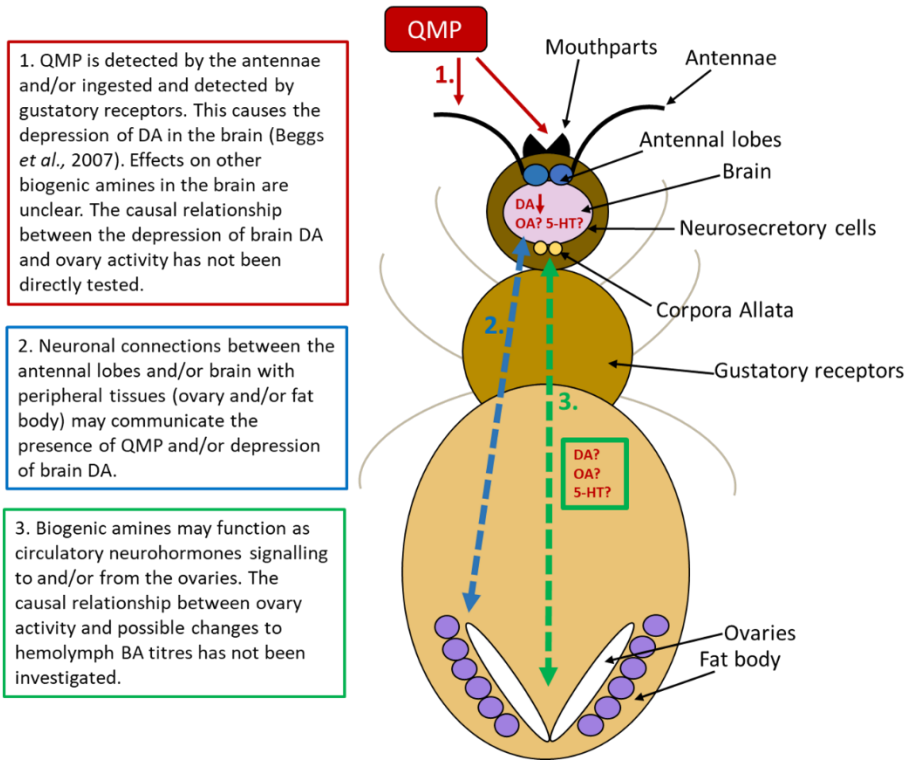

Figure 1.7 Diagram showing the causal relationships among QMP, DA/ other biogenic amines and ovary activation. Dotted lines indicate an uncertain causal relationship, while solid lines indicate a proven causal relationship.

additional function of QMP as an agent of reproductive control produced by honey bee queens.

1.6 Research Aims

By investigating the hypothesis that QMP exploits aminergic signalling pathways to cause sterility in workers, this research aims to improve our understanding of the mechanisms by which this highly derived queen pheromone induces reproductive constraint in honey bees. Empirical testing of if, and how, QMP chemically suppresses worker reproduction is central to our understanding of how the pheromones mediating reproductive division of labour in eusocial insect societies function: as agents of control, honest signals of fertility, or a combination of both.

In Chapter three, I attempt to elucidate how changes in biogenic amines in the brain are linked with ovary development by investigating a possible pathway by which they could

signal to the ovary. Specifically, I test the hypothesis that biogenic amines function as circulatory hormones in honey bees that are capable of interacting directly with ovarian tissue. In addition, the use of laboratory microcolonies of workers as a proxy for studying whole hives is established.

In Chapter four, I directly test the causal relationship between dopamine titres and ovary development through a series of manipulative experiments using dopamine supplementation and inhibition on laboratory microcolonies. This is to test the hypothesis that QMP's modulations to dopamine levels are a driver of worker reproductive constraint.

Given the idea that QMP acts as a suppressive agent to induce sterility in her workers, I address the inherent hypocrisy of how the queen's reproduction is not inhibited by her own QMP in Chapter five. I test one possible mechanism by which QMP could hypothetically serve as a repressive agent in workers but not queens, and speculate at other mechanisms that could be investigated further.

In Chapter six, I apply my understanding of QMP's effects on biogenic amine signalling pathways and investigate how resilient this delicately balanced system is to external environmental stressors. I test the effects of a commonly used pesticide, imidacloprid, on worker responses to QMP at molecular, physiological and behavioural levels, and hypothesize implications for whole-colony function.

Finally, in Chapter seven I bring together my findings with the wider literature and speculate as to the specific mechanisms by which QMP may regulate reproduction and identify key avenues for future research.

Chapter 2

General Methods

2.1 Honey bee maintenance

2.1.1 Laboratory microcolonies

Apis mellifera colonies were kept according to standard beekeeping practices in British National hives at the University of Leeds School of Biology Research Apiary. To set up laboratory microcolonies, frames of emerging brood were collected from three source hives and incubated overnight at 35°C. The following day, newly emerged workers were transferred into metal cages with a glass sliding door and holes for insertion of food caps and a water tube (10 x 10 x 5.5 cm, Small Life Supplies UK; Figure 2.1). 80-100 bees were randomly allocated to each cage, and cages were randomly assigned to treatments. Cages were maintained in the dark at 35°C and 20-40% relative humidity (RH). Caged experiments were limited to 10 days in duration. After this point there were large drop-offs in survival as bees reached maximum gut capacity due to their inability to exhibit natural defecation behaviours (personal observation).

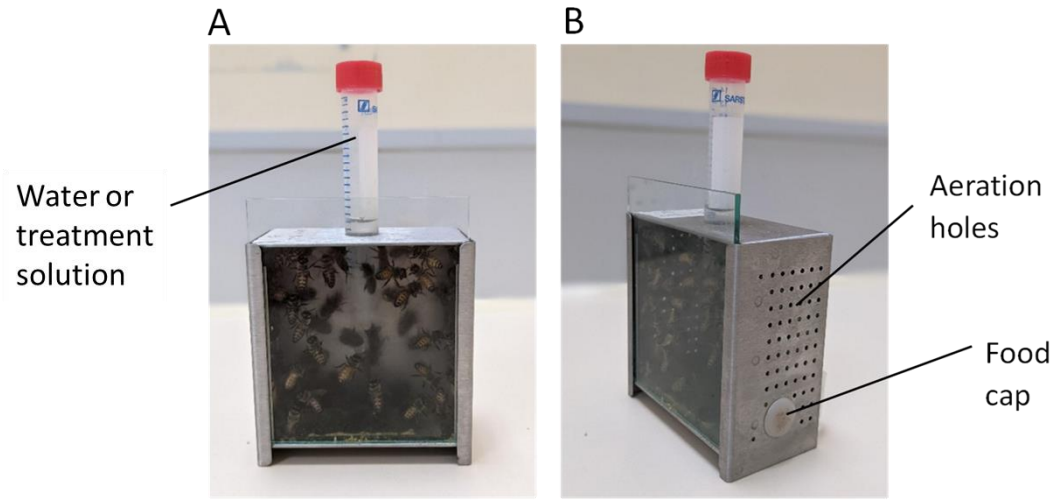

Figure 2.1 Honey bee cages Metal cages used to house honey bees in laboratory experiments, showing **A.** falcon tube used to hold water or treatment solutions, **B.** food cap and aeration holes. Photograph taken by Jessica Bouwer (MSc student) and used with permission.

Cages were monitored daily for dead bees, which were recorded and discarded. All cages were fed complete bee food (CBF), a high protein diet formulated to permit ovary activity (Duncan *et al*., 2016) consisting of: 20g pollen, 52g sucrose, 18.8g brewer's yeast and 9.2g lactalbumin. CBF was prepared in large batches and stored at -20°C. Cages received 2 g of CBF mixed with honey to form a paste each day. Food and water (or treatment solution) intake were recorded daily for each cage to determine whether experimental treatments were associated with changes in appetite or treatment solution drinking rates that could confound or exacerbate the results of any given experiment. Mean food and water consumed per bee per day was calculated by subtracting the weight of food/water out by that of the food/water given the previous day and dividing by the current number of bees in the cage. In the rare occurrence of obvious food and water tube leakages, these measurements were excluded from the dataset.

2.1.2 QMP administration

Synthetic QMP used in laboratory experiments (supplied by Intko Supply Ltd, Canada) contained the five major QMP components: (E)-9-oxodec-2-enoic acid (9-ODA), both enantiomers of (E)-9-hydroxydec-2-onoic acid (9-HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA). QMP is quantified in 'queen equivalents' (QE), where 1 QE is the mean quantity of QMP found in the mandibular glands of a laying queen. 1 QE consists of 150 µg 9-ODA, 55 µg 9-HDA, 13 µg HOB, and 1.5 µg HVA (Slessor *et al*., 1988). QMP was dissolved in ethanol at a concentration of 0.01 QE/µL (queen equivalents per microliter) and stored at -20°C. queenright (QR) laboratory microcolonies were exposed to QMP by pipetting a 10 µL droplet of 0.01 QE/µL onto a glass slide and placing this slide within the cage. This amounts to a QMP exposure of 0.1 QE per cage per day (0.01 QE/µL x 10 µL). Laboratory microcolonies maintained in queenless (QL) condition received 10 µL ethanol as a solvent control each day. Slides were replaced daily. QR and QL microcolonies were maintained in separate identical incubators to avoid cross-contamination of QMP components.

2.2 Ovary dissection and scoring

To assess ovary activity in caged experiments, bee ovaries were dissected and imaged on day 10. Cages of bees were incubated at 4°C prior to dissection to cold-anesthetise them. Images of dissected pairs of ovaries were captured at 2.5 x magnification using GXCaptureT software and a GXCAM-U3 camera mounted on a GXMXTL3101 stereomicroscope.

Images were later scored for activity level using a modified Hess scale as in previous work (Hess, 1942; Duncan *et al*., 2016). Figure 2.2 depicts the key morphological features associated with each stage of ovary development. Ovaries that are thin and lacking signs of cell differentiation are scored as a zero, ovaries that are slightly thickened with signs of differentiated cells but no deposition of yolk are scored as a one, ovaries with clearly defined oocytes and yolk deposition are scored as a two, and ovaries with at least one fully mature oocyte are scored as a three (Figure 2.2). Where possible, scoring was carried out by three independent scorers who had been blinded to treatment. The final

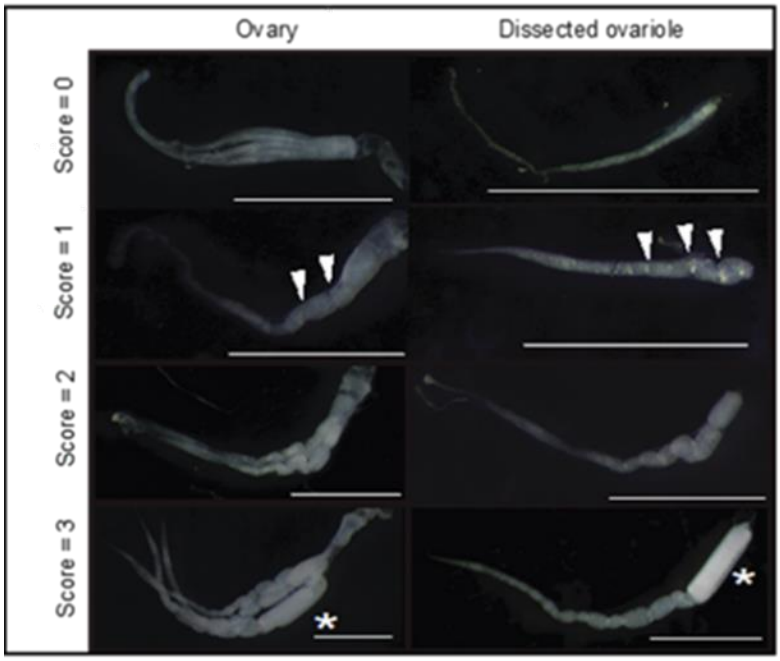

Figure 2.2 Morphology of ovaries and single ovarioles in each ovary score category showing the four distinct stages of ovary activity. Mature oocytes are denoted by asterisks, while arrows mark signs of differentiation. Scale bars indicate 1 mm. Figure provided by Dr Elizabeth J. Duncan and used with permission.

score used was the mode of each three scores. In the rare occurrence that all scorers disagreed, the score of the most experienced scorer was used. If only one scorer was available, images were scored by one person (myself) using 'blinder' software to blind the scorer to treatment and randomise the order of images (Cothren *et al*., 2018).

2.3 Measurement of biogenic amines using HPLC-FLD

2.3.1 Sample collection

Brain, ovary and hemolymph samples were often collected from workers following a range of experimental treatments for analysis of biogenic amine content. Workers were anesthetised by incubating whole microcolonies at 4°C prior to sample collection. 1 µL hemolymph was extracted from the abdomen of each bee using a pulled glass capillary needle. Whole brains were dissected, with care taken to remove hypopharyngeal glands, optic and antennal lobes and any connective tissue to avoid contamination of brains with biogenic amines from other sources. Ovary pairs were dissected and scored immediately, as capturing images for later blind-scoring would have delayed tissue collection and resulted in degradation of biogenic amines. All samples were immediately snap frozen in liquid nitrogen then stored at -80°C until later analysis of biogenic amine content using HPLC-FLD.

2.3.2 Preparation of samples and standards

Samples were prepared for HPLC-FLD analysis as follows. Brain and ovary samples were homogenized on ice in 150 µL ice-cold 0.1M perchloric acid (PCA) for one minute using a micropestle fixed to a pellet-pestle cordless motor (Kimble-Chase). 1 µL Hemolymph samples were added to 149 µL ice-cold 0.1M PCA (bringing the total sample volume up to 150 µL) and vortexed for 30 seconds. On the rare occasion that under 1µL hemolymph had been collected, the volume of 0.1M PCA added was increased as required to bring the total volume up to 150 µL, and adjustments to final biogenic amine content calculations were made to account for this. All samples were centrifuged at 13,000 RPM at 4°C for 30 minutes to remove cellular debris. The supernatant was purified of small

particulates prior to HPLC-FLD by centrifuging for a further five minutes through a filter column (0.45 µm, Thermo Scientific), then transferred into a 250 µL HPLC micro vial (Agilent Technologies) and refrigerated until HPLC-FLD analysis later that day. Samples were prepared simultaneously in batches of 12 or 24. The preparation batch of each sample was noted and included as a random effect during data analysis.

External standards of norepinephrine, dopamine, octopamine, serotonin and tyramine were prepared at 0.025, 1, 10 and 50 µM concentrations. Standards were prepared in ice-cold 0.1M PCA from stocks stored at -80°C on each day of sample preparation and refrigerated until same-day HPLC-FLD analysis alongside samples.

2.3.3 HPLC-FLD system

The simultaneous determination of biogenic amines in biological samples was achieved using high performance liquid chromatography separation with fluorescent detection (HPLC-FLD) and pre-column derivatization (Figure 2.3). The HPLC-FLD instrument was based in the School of Chemistry HPLC Facility at the University of Leeds and operated by Jeanine Williams. The instrument was an Agilent 1290 Infinity II UHPLC system consisting of a multisampler (P.N. G7167B), 1290 high speed pump (P.N. G7120A), and 1290 FLD spectra (P.N. G1321B). The column used for separation of biogenic amines by their retention times was an Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 50 mm, 1.9 um, P.N. 699675-902) maintained at 40°C ± 0.8°C in a 1290 MCT column compartment (P.N. G7116B).

Mobile phase A contained 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$ and 5 mM NaN_3, adjusted to pH 8.2 using concentrated HCl and filtered and degassed under vacuum (Thermo Scientific Nalgene rapid-flow bottle filter, pore size 0.2 µm). Mobile phase B contained acetonitrile: methanol: water in a ratio of 45:45:10 (v:v:v). The injection diluent was prepared by adding 0.4 mL concentrated H_3PO_4 to 100 mL of mobile phase A. OPA and Borate buffer were used as derivatization reagents and were supplied as ready-made solutions by Agilent (Borate buffer: 0.4 M in water, pH 10.2, 100 mL, P.N. 5061-3339; OPA: 10mg/mL in 0.4M borate buffer and 3-mercaptoproprionic acid, supplied in 1mL

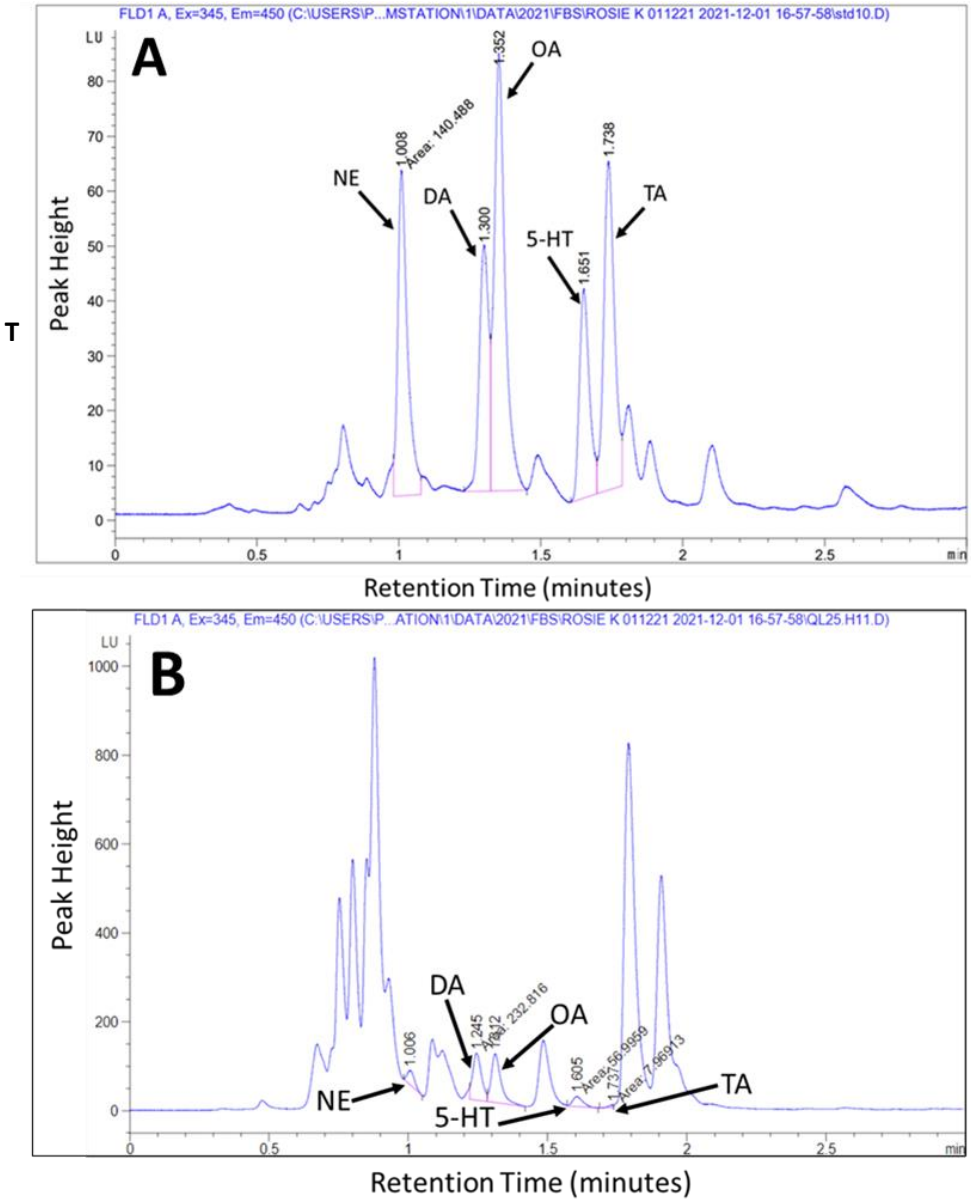

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Figure 2.3 HPLC-FLD chromatograms showing the simultaneous determination of the biogenic amines norepinephrine (NE), dopamine (DA), octopamine (OA), serotonin (5-HT), and tyramine (TA) in **A)** a 10 µM mix of standards and **B)** a sample of honey bee hemolymph (prepared as described in Section 2.3.2).

ampoules, P.N. 5061-3335). The injection diluent and OPA derivatization reagent were stored at 4°C. Details of the injection program used can be found in Table 2.1. Mobile phase A and B were used in gradient as described in Table 2.2. The flow rate was kept constant at 0.5 mL/min.

Step	Action
1.	Draw 1.5 µL from Borate buffer vial
2.	Draw 2.5 µL from sample vial
3.	Mix 4µL in air default speed 5 times
4.	Wait 0.2-minutes
5.	Draw 1 µL from OPA vial
6.	Mix 5 µL in air, 10 times default speed
7.	Draw 5 µL from injection diluent vial
8.	Mix 10 µL in air default speed 8 times
9.	Inject
10.	Wait 0.1-minute
11.	Valve bypass

Table 2.1 Details of HPLC-FLD injection program

Time (min)	% A	% B
0	98	2
0.5	57	33
3	5	95
3.1	0	100

Table 2.2 Details of the mobile phase gradient used for HPLC-FLD

Detection of separated biogenic amines was achieved using fluorescence detector settings of 345 nm (excitation wavelength) and 450 nm (emission wavelength). Chromatograms produced from detector signals over three minute sample run durations were recorded, integrated and analysed using Agilent OpenLab CDS software, ChemStation Edition (Rev C.01.10 [30]) with Agilent OpenLab Intelligent Reporting (5.0.0.352). Standard curves were generated for each biogenic amine by plotting their peak area at each concentration and fitting a linear regression line (see Appendix A, Figure A.1). Biogenic amine concentrations in samples were quantified from their peak areas using the equations of the lines fitted to the standard curves. Brain, ovary and

hemolymph biogenic amine levels levels are expressed as pmol/brain, pmol/ovary and pmol/µL respectively.

2.4 Data analysis

All data analysis was carried out in R version 4.1.2 (R Core Team, 2021). Paired T-tests were carried out using the *rstatix* package (Kassambara, 2021). Linear mixed effects models (LMMs) and generalised linear mixed effects models (GLMMs) were built using the package *lme4* (Bates *et al*., 2015). The packages *survival* and *coxme* were used to construct Cox proportional hazards models (CPH) with mixed effects (Therneau, 2020; Therneau, 2021), and cumulative link mixed models (CLMMs) were carried out using the package *ordinal* (Christensen, 2019). *Post-hoc* testing was carried out on models using the package *emmeans* (Lenth, 2022). Graphs were prepared using *ggplot2* (Wickham, 2016) and *ggpubr* (Kassambra, 2020).

Chapter 3

Transmission of QMP's signal to the ovary

3.1 Introduction

In honey bee hives, queen mandibular pheromone (QMP) is one of the major factors inhibiting worker reproduction (Hoover *et al*., 2003). In the presence of QMP, worker ovarian development is actively inhibited by Notch signalling (Duncan *et al*., 2016), and possibly apoptosis of oocytes that do develop (Ronai *et al*., 2015). In the absence of QMP worker ovaries are completely remodelled, oogenesis is initiated (Duncan *et al*., 2016, 2020), and workers lay haploid eggs destined to become drones or male bees (Jay, 1968). Despite decades of research, the processes acting upstream of these mechanisms in the ovary and their integration with the signal from QMP are still uncertain (Section 1.5.2; Figure 3.1).

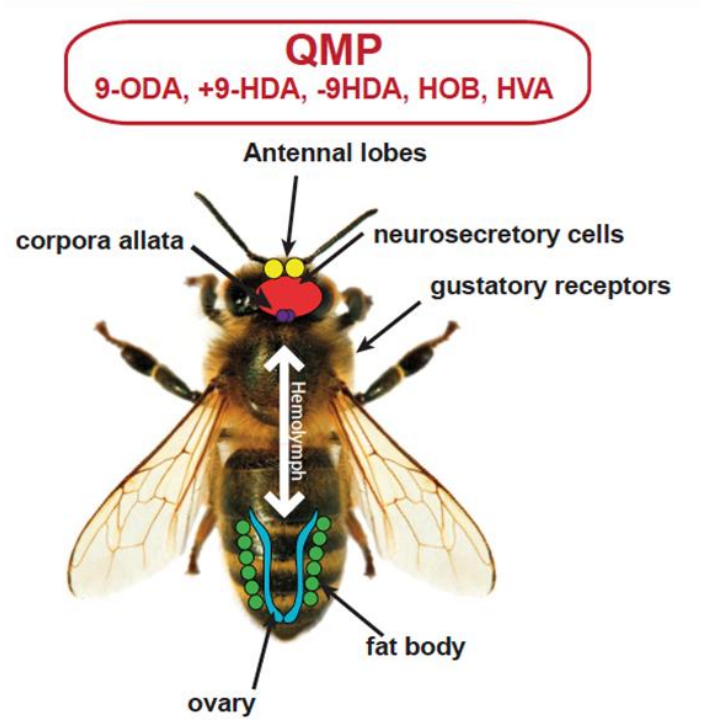

Figure 3.1 Transmission of QMP's signal to the ovary. Honey bee reproduction in workers is repressed, at least in part, by QMP. Workers are presumably exposed to QMP via the antennal lobes or gustatory receptors, and this signal is transmitted to the ovary to repress oogenesis. How QMP's signal is transmitted to the ovary represents a key gap in our knowledge.

Secreted from the queen's mandibular gland, QMP is picked up by young workers attending the queen and spread among colony members through worker-worker contact and trophallaxis (Naumann *et al*., 1991; Watmough, 1997). QMP's signal is then transmitted to the ovary, where active Notch signalling inhibits ovary activity by blocking oogenesis (Duncan *et al*., 2016). Although the processes linking the detection of QMP with this ovarian response have not been fully elucidated, biogenic amines have been widely implicated in playing a role (refer section 1.5.3, General Introduction).

Through their diverse functions as neurotransmitters, neuromodulators and neurohormones (Evans, 1980), biogenic amines such as dopamine, octopamine, tyramine and serotonin coordinate a range of behavioural and physiological processes in insects (reviewed by Sasaki & Harano, 2010). In eusocial insects, it has been proposed that biogenic amines have been co-opted into regulating elements of division of labour and control of worker reproduction (reviewed by Sasaki *et al*., 2021).

In honey bees, octopamine is involved in coordinating task polyethism by mediating the behavioural maturation from nurse to forager (Schulz *et al*., 2002; Schulz & Robinson, 2001; Wagener-Hulme *et al*., 1999), while dopamine and tyramine are thought to be involved in regulating ovarian development (Linn *et al*., 1994; Salomon *et al*., 2012; Ken Sasaki & Harano, 2007). In queen-less (QL) workers, brain dopamine levels correlate positively with ovary developmental stage (Harris & Woodring, 1995; Sasaki & Nagao, 2001), and supplementary feeding of dopamine (Dombriski *et al*., 2003) and tyramine (Salomon *et al*., 2012) to QL worker honey bees further enhances ovary activity levels. Importantly, QMP also directly depresses brain dopamine through the component HVA (Beggs *et al*., 2007; Figure 1.6). Exposure to HVA alone can reproduce some of QMP's inhibitory effects on worker reproduction (Princen *et al*., 2019), implying there may be a link between dopamine and the regulation of ovarian development.

Little is known about the processes linking changes in brain biogenic amine titres with the mechanisms constraining oogenesis within the ovary (Notch signalling: Duncan *et al*. 2016; Apoptosis: Ronai *et al*. 2015). Crucially, multiple biogenic amine receptors are

expressed in the ovarian tissue, including the dopamine receptors AmDop1 and AmDop3, the octopamine receptor AmOA1 and the serotonin receptor Am5-HT7 (Vergoz *et al*., 2012), implying that direct interaction of these biogenic amines with the ovary is possible. However, the mode of transportation of biogenic amines to the ovary has not been investigated. Given that insects have an open circulatory system, peripheral organs would be directly exposed to any compounds present in the hemolymph. Biogenic amines are present in the hemolymph of a number of insect species. For example, several biogenic amines, including dopamine and octopamine, have been detected in the hemolymph of male cabbage looper moths (Linn *et al*., 1994), and hemolymph dopamine titres in the silkworm *Bombyx mori* mediate the onset of reproductive diapause in adulthood (Noguchi & Hayakawa, 2001). In honey bee males, hemolymph dopamine titres change in parallel with brain dopamine titres, and hemolymph dopamine is thought to interact directly with aminergic receptors on the testes to modulate mating flights and copulation (Akasaka *et al*., 2010; Sasaki & Watanabe, 2022). However, little is known concerning biogenic amines in honey bee worker hemolymph, and whether they are altered by QMP in parallel to the brain.

Here, it is hypothesised that biogenic amines act as QMP-responsive circulating neurohormones in the hemolymph and interact directly with biogenic amine receptors in the ovary to mediate reproductive constraint. To test this hypothesis, levels of dopamine, octopamine and serotonin in the hemolymph and ovaries of workers from experimental queen-less (QL) and queen-right (QR) hives were measured. To understand the interplay between hemolymph biogenic amine titres and previously reported effects of QMP on brain biogenic amines (Beggs *et al*., 2007; Harris & Woodring, 1995), brain titres were also examined. This chapter aims to bridge the gap in our understanding of the link between QMP's effects on biogenic amines in the brain with its command of worker ovarian physiology.

3.2 Methods

3.2.1 Generation and maintenance of QR and QL hives

A. mellifera colonies were kept according to standard beekeeping practices in poly nucleus hives at the University of Leeds School of Biology Research Apiary. Genetically related queenless (QL) sister hives were generated from queenright (QR) hives in July-September 2020 by transferring frames of emerging brood into a single poly nucleus box. To prevent workers rearing a new queen from any queen-laid eggs from the brood frames, QL hives were monitored bi-weekly for queen cells, which were destroyed. Workers were routinely sampled from QR and QL hives to assess levels of ovary activity as further confirmation of queen-status of the colonies. It was expected that no workers in QR hives would have active ovaries (where ovaries are considered 'active' at scores of 2 or above; Figure 2.2), while in QL hives around 24-30% of workers will activate their ovaries (Jay, 1968; Miller & Ratnieks, 2001).

3.2.2 Mark-release-recapture experiment

Workers sampled for tissue collection from QL and QR hives were age-matched to control for age-related variations in biogenic amine titres (Harris & Woodring, 1992). Age-matching was achieved using mark-release-recapture. Frames of emerging brood were collected from three source hives and incubated at 35°C. All bees emerging within 24 hours were marked with a coloured dot on their thorax using a POSCA non-toxic paint marking pen, then returned to either the experimental QL or QR hive (Figure 3.2). With a view to resample equal numbers of workers with each ovary score from experimental hives, workers were returned to QR and QL hives in a ratio of approximately 1:3 respectively, as QL workers display substantial variation in ovary score as some bees readily activate their ovaries in response to queen absence (Miller & Ratnieks, 2001). Three marking trial replicates were carried out between August-September 2020, with a different paint colour used on each day. In trial one, 147 and 82 marked bees were released in QL and QR hives respectively. In trial two, respective numbers released were 267 and 123, and in trial three, 304 and 150.

Figure 3.2 Paint-marked bees Image of paint marked bees being released into an experimental hive

Marked bees were resampled from hives at two timepoints after release: 10 and 21 days. Day 10 was selected as a time-point as a direct age-based comparison with laboratory experiments, which are terminated on day 10 (Section 2.1.1, General Methods). Additional workers were sampled on day 21 as ovary activation was observed to be slower in the hive compared with the laboratory, possibly due to differences in nutrition (caged workers are fed a high protein diet specifically formulated to permit ovary activity; Refer section 2.1.1, General Methods). This second later timepoint allowed sufficient time for workers to reach later states of ovary maturity (i.e. scores of 2 and above; Koudjil & Doumandji, 2008) and enable physiologically similar comparisons with caged honey bee workers.

Brain, ovary and hemolymph samples were collected from workers captured from each time point for later measurement of biogenic amines as described in Section 2.3.1. Samples were prepared as described in Section 2.3.2 and HPLC-FLD was used to quantify

octopamine, dopamine and serotonin as described in Section 2.3.3. Numbers of workers sampled at each time point in each replicate is found in table 3.1.

3.2.3 Establishing laboratory microcolonies

QR and QL microcolonies were setup in cages in the laboratory to; 1. Isolate QMP's effects from other pheromones and behaviours in the hive and 2. Validate the laboratory system as a model for the hive. Microcolonies of 100 workers were set up and maintained in cages as described in section 2.1.1. Microcolonies were maintained in QR condition (i.e. with synthetic QMP) or QL condition (i.e. with solvent control) as described in section 2.1.2. Three replicates of each microcolony were set up in September 2021 within three days of each other. On day 10, brain and hemolymph samples were collected for later analysis of biogenic amine content from ten randomly selected bees per cage as described in Section 2.3.1. Samples were prepared as described in Section 2.3.2 and HPLC-FLD was used to quantify octopamine, dopamine and serotonin as described in Section 2.3.3.

To validate the efficacy of synthetic QMP's inhibition of reproduction in QR microcolonies and assess ovary activation rates in QL microcolonies, the ovaries of 20-30 randomly selected bees from each cage were also dissected on day 10. Dissected ovaries were imaged and scored blinded to treatment as described in Section 2.2.

3.2.4 Data analysis

Data analysis was carried out in R. Details of packages used for analysis are found in section 2.4.

Differences in ovary activity between QR and QL workers were analysed using Pearson's Chi Squared tests. In bees resampled from the hive for tissue collection, ovary activity was compared across four conditions: QR day 10, QL day 10, QR day 21 and QL day 21. In the laboratory experiment, ovary scores were compared between QR and QL microcolonies at day 10.

Levels of the biogenic amines dopamine, octopamine and serotonin in tissues of workers sampled from the hive were each analysed using linear mixed-effects models (LMMs) or generalized linear mixed-effects models (GLMMs). LMMs were fitted for each biogenic amine and each tissue, with time-point (ie. day 10 or 21), colony status (i.e. QR or QL) and their interaction as fixed effects and marking replicate and HPLC-FLD analysis batch as random effects. Normality and homoscedasticity of model residuals were tested by visual examination of qqplots, histograms and predicted vs fitted values. If these assumptions were not met, GLMMs with an inverse link function were fitted, the residuals of which met the assumptions of normality and homoscedasticity (i.e. GLMMs were used for analysis of brain dopamine, brain octopamine, hemolymph octopamine and ovary dopamine). P-values for fixed effects were obtained by comparing the likelihood ratio of the maximal model to that of the model without the fixed effect of interest (Bates *et al*., 2015). If fixed effects were statistically significant, *post-hoc* pairwise testing was carried out on the maximal model using estimated marginal means. P-values were Tukey-adjusted to control for multiple testing.

In the laboratory experiment, differences in brain and hemolymph levels of dopamine, octopamine and serotonin between QR and QL microcolonies were each analysed using Mann Whitney-U tests. Prior to this, Shapiro-Wilk and Bartlett tests revealed deviations from normality and homogeneity of variance respectively for each biogenic amine in each tissue measured.

To determine whether biogenic amine titres in the brain, hemolymph and ovary correlate with ovary activity in QL workers, analysis of biogenic amine levels across each ovary score was carried out for workers from the QL laboratory microcolony using GLMMS. GLMMs with an inverse link function were fitted for each biogenic amine and each tissue, with ovary score as a fixed effect and replicate and HPLC-FLD analysis batch as random effects. As above, LMMs were initially fitted but failed to meet assumptions of normality and homoscedasticity. Residual plots of GLMMs were visually inspected and revealed no obvious deviations from these assumptions. The exception is hemolymph octopamine, for which a log link function was applied as this was deemed to be a better

fit for the data following visual assessment of model residual plots. The overall significance of ovary score was computed by comparing the likelihood ratio of the full model to that of a null model containing random effects only (Bates *et al*., 2015). *Post-hoc* testing was carried out using estimated marginal means with Tukey adjustment.

3.3 Results

3.3.1 Recapture success rate

Of the 1,093 bees marked and released in total across all three trials, just 86 were successfully recaptured, a recapture rate of 7.9%. Due to this low success rate, only 8 QR workers were recaptured in total for the 21-day timepoint, hence the lower n values in this group. A breakdown of the numbers of marked bees released and recaptured from experimental hives in each trial is displayed in Table 3.1.

	Released		**Recaptured (Day 10)**		**Recaptured (Day 21)**		**Total Recaptured**		**%Recapture Success**		**Overall % Recapture Success**
Trial	QL	QR	QL	QR	QL	QR	QL	QR	QL	QR	
1	147	82	10	11	9	7	19	18	12.9%	22.0%	
2	267	123	10	6	17	1	27	7	10.1%	5.7%	
3	304	150	10	6	0	0	10	6	3.3%	4%	
Total	738	355	30	24	26	8	56	31	7.6%	8.7%	7.9%

Table 3.1 Numbers of marked bees released into and recaptured from experimental hives at each timepoint. Total bees recaptured at each time point represents n values for each group.

3.3.2 Ovary activity of QR and QL workers

As expected, workers responded to the absence of a queen (or synthetic QMP) by activating their ovaries (Figure 3.3). A higher degree of ovary activity was observed in QL relative to QR workers in both hive and laboratory microcolonies, though this was statistically significant only in the laboratory (lab: $\chi^2 = 35.92$, df = 3, $p < 0.001$; hive: $\chi^2 = 9.20$, df = 9, $p = 0.4192$). Repression of ovary activity was to a lesser degree in QR laboratory microcolonies maintained with synthetic QMP than by a real queen in the

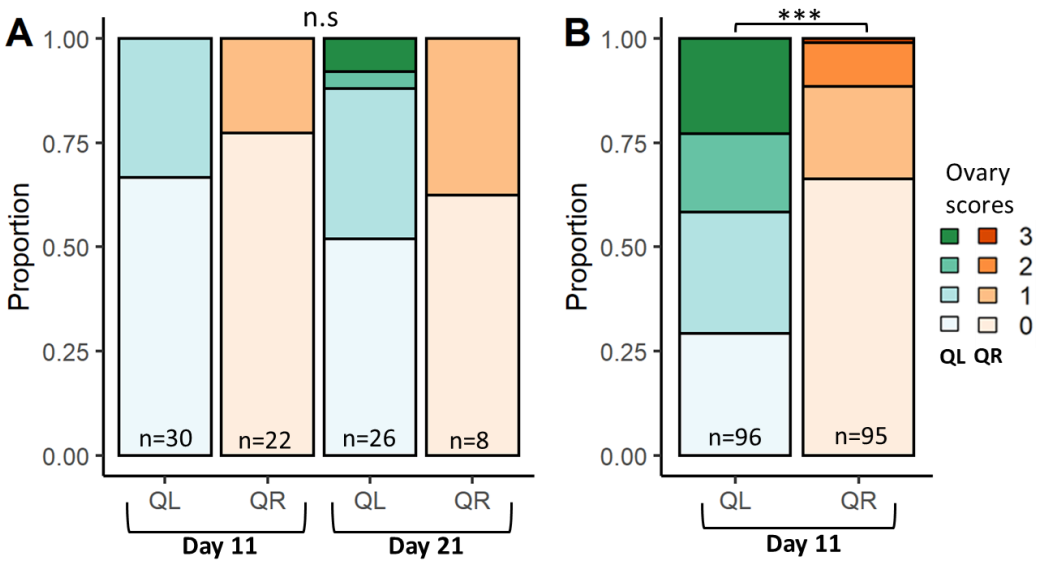

Figure 3.3 Ovary activation rates in the hive and laboratory microcolonies. A. Ovary activity was measured in marked workers released and recaptured from queenless (QL – shades of green) or queenright (QR - shades of orange) experimental hives at 10 and 21 days old. **B.** Comparative ovary activation rates of workers from laboratory-maintained micro-colonies. 'QR' microcolonies were supplied with 0.1 QE of synthetic QMP to emulate queenright conditions. 'QL' microcolonies received just a solvent control to emulate queenless conditions. Ovary scores range from 0-3 (Figure 2.2), where a higher score (darker shade) relates to a higher degree of ovary development. Ovary activity is presented as the proportion of workers with each ovary score within each group. Numbers of workers in each group (n) is displayed at the base of each bar. Differences in ovary activation rates between groups were determined using Pearson's Chi Squared, statistical differences ($p<0.001$) are denoted by asterisks. In the hive **(A)**, ovary activation rates did not differ between QL and QR hives on the days sampled, whereas ovary activity levels were higher in QL than QR microcolonies by day 10 in the laboratory **(B)**.

hive, as a small number of workers with active ovaries (11.58%) were observed in the former but not the latter (where 'active ovaries' are scores of 2 or above; Figure 2.2).

In addition, ovary activation rates of QL workers were accelerated in laboratory microcolonies compared with that seen in the hive. In QL workers sampled from the hive, ovary activation (scores of 2 or above; Figure 2.2) was not observed until day 21, while 42% of their laboratory-maintained counterparts had activated their ovaries by day 10. Due to the lower than expected ovary activity rates in the hive along with low recapture success rates, only a limited number of QL workers representative of each

ovary score could be obtained. A meaningful analysis of the differences in biogenic amines between ovary scores of QL workers could therefore not be carried out for workers sampled from the hive, and was carried out only within laboratory microcolonies.

3.3.3 Serotonin is unaffected by QMP

Serotonin could not be consistently detected at measurable quantities in the hemolymph or ovary, hence only brain data is presented (Figure 3.4, Figure 3.5).

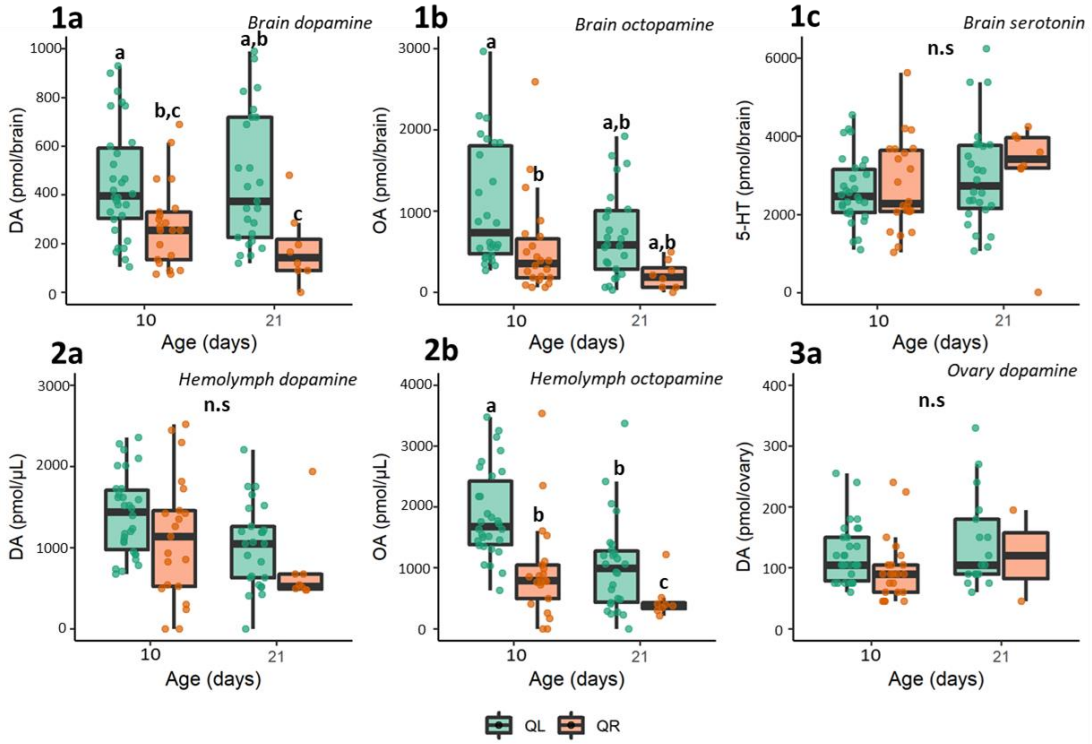

Figure 3.4 Biogenic amine levels in workers from QR and QL hives. Levels of biogenic amines in **1.** brain, **2.** hemolymph and **3.** ovary tissue of marked workers released and recaptured from queenless (**QL** - green) or queenright (**QR -** orange) hives at 10 and 21 days old. As in Table 3.1, N = 30 (QL day 10), 24 (QR day 10), 26 (QL day 21) and 8 (QR day 21) for each tissue. Biogenic amines **a.** dopamine **(DA), b.** octopamine **(OA)** and **c.** serotonin **(5-HT)** were quantified in parallel using HPLC-FLD. Serotonin was not detected in the hemolymph so is not shown. Dopamine was the only biogenic amine detected in the ovary. For data visualisation clarity, outliers lying beyond the range of both boxplots were not plotted, but were included in all analyses. Biogenic amine contents of tissues was analysed using LMMs and GLMMs (depending on normality) with *post-hoc* testing carried out using estimated marginal means. Significant pairwise comparisons to the level of $p < 0.05$ and bellow are denoted by boxplots not sharing a letter. Non-significant pairwise comparisons are denoted by 'n.s'. For table of *post-hoc* comparisons see Appendix B, Table B.1

Although detected in the brain, serotonin was not affected by QMP exposure. In the hive, levels of serotonin in worker brains were unaltered by queen presence, age or the interaction of these factors (Figure 3.4; LMM; Queen status: $\chi^2 = 0.69$, df = 2,83, p = 0.71; Age: $\chi^2 = 1.39$, df = 2,83, p = 0.50; Interaction: $\chi^2 = 0.28$, df = 1,84, p = 0.60). Similarly, no differences in brain serotonin were observed between QL and QR workers in laboratory microcolonies (Figure 3.5; MWU; W = 395, df = 56, p = 0.703), suggesting no direct effects of QMP exposure on serotonin.

3.3.4 Brain dopamine correlates with ovary activity

An increase in brain dopamine was associated with increasing ovary activity in QL laboratory microcolonies (Figure 3.6; GLMM; $\chi^2 = 8.53$, df = 3,24, p <0.05). Workers with signs of yolk deposition (ovary score of 2; Figure 2.2) had significantly elevated brain dopamine levels compared with less active workers (ovary score of 1; Z = 3.19, p <0.01) Interestingly, in workers possessing ovaries with fully mature oocytes (ovary score of 3; Figure 2.2) brain dopamine levels returned to that seen prior to the initiation of oogenesis (scores of 0 and 1; Figure 2.2), consistent with a role for dopamine early in the process of ovary activation. For a complete list of all *post-hoc* pairwise comparisons between ovary scores, see Table 3.2 and Figure 3.6.

Brain dopamine levels were also depressed in the presence of QMP. In the hive, brain dopamine levels were significantly lower in QR workers (Figure 3.4; GLMM; $\chi^2 = 18.58$, df = 2,80, p <0.001), while both age its interaction with colony queen status had no effect

Brain dopamine post-hoc pairwise comparisons					
Contrast (ovary scores)	Estimate	SE	df	Z ratio	P value
0-1	-0.000157	0.000335	Inf	-0.457	0.9682
0-2	0.000652	0.000324	Inf	1.973	0.1981
0-3	0.000124	0.000362	Inf	0.335	0.9870
1-2	0.000809	0.000255	Inf	3.186	0.0079**
1-3	0.000280	0.000283	Inf	0.993	0.7533
2-3	-0.000529	0.000306	Inf	-1.733	0.3065

Table 3.2 *Post-hoc* comparisons of brain dopamine levels in QL workers between all pairwise combinations ovary scores. *Post-hoc* testing was computed using estimated marginal means on the maximal GLMM. GLMMs were fitted with inverse link functions, hence contrast estimates are on the inverse scale. P-values are Tukey adjusted.

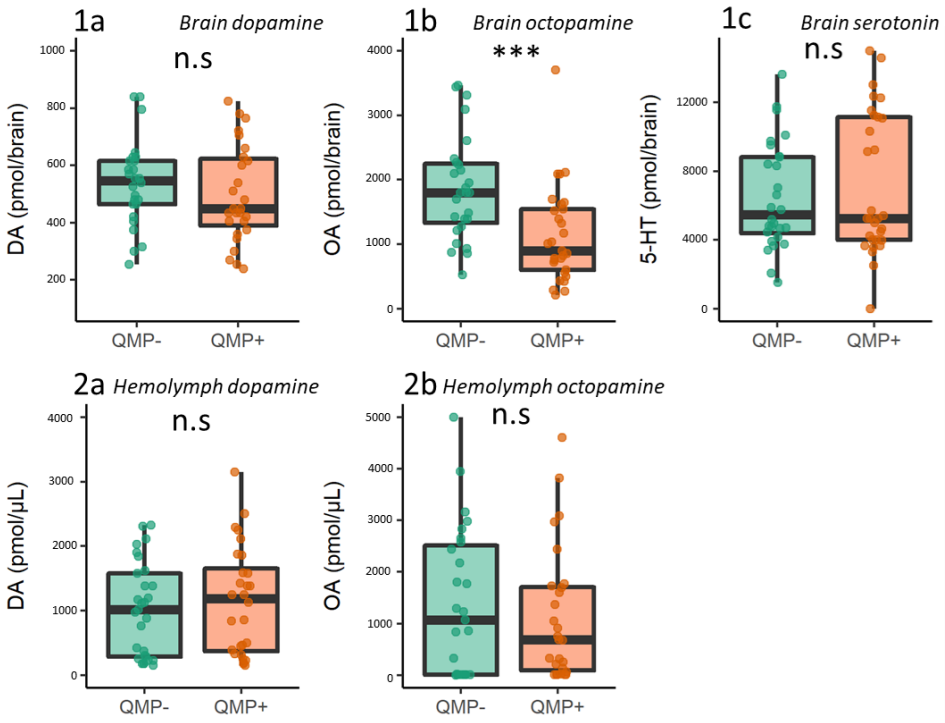

Figure 3.5 Biogenic amine levels in workers from QR and QL laboratory microcolonies. HPLC-FLD quantification of the biogenic amines **a.** dopamine (DA), **b.** octopamine (OA) and **c.** serotonin (5-HT) in **1.** brain and **2.** hemolymph tissue of 10-day old laboratory-maintained workers. 5-HT was not detected in the hemolymph so is not shown. Workers were maintained in laboratory microcolonies of 100 bees. QMP+ microcolonies (orange) were supplied with 0.1 QE of synthetic QMP to emulate queenright hive conditions. To emulate queenless hive conditions, QMP- microcolonies (green) received just a solvent control. There were three replicates of each microcolony and brain tissue was obtained from 10 randomly selected bees from each (total n = 30 for each treatment). For clearer data visualisation, outliers lying beyond the range of both boxplots are not plotted, but are included in all data analyses. Statistical significance (Mann-Whitney U) at the level of $p<0.001$ is denoted by asterisks, and "n.s" denotes statistical non-significance.

(GLMM; Age: $\chi^2 = 2.27$, df = 2,80, p = 0.32; Interaction: $\chi^2 = 1.88$, df = 1,81, p = 0.17). *Post-hoc* testing revealed the depression of brain dopamine in QR workers to be statistically significant at both sampling timepoints (Day 10: Z = -2.84, $p < 0.05$; Day 21: Z = -2.740, $p < 0.05$; Complete list of pairwise comparisons displayed on Figure 3.4 and Appendix B, Table B.1). However, in QR laboratory microcolonies there were no significant reductions of brain dopamine levels compared with QL workers (Figure 3.5; MWU; W = 473.5, df = 56, p = 0.41), despite the observed inhibition of ovary activity by synthetic QMP (Figure 3.3).

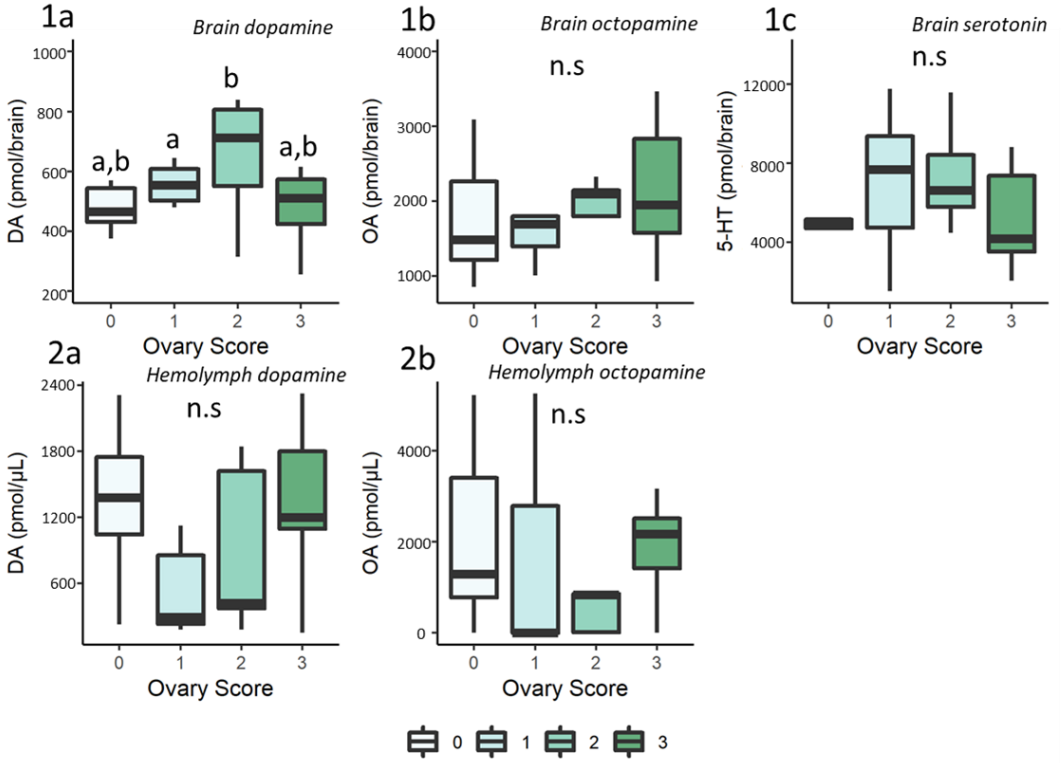

Figure 3.6 Brain biogenic amine titres vary with ovary activity level of QL workers. HPLC-FLD quantification of the biogenic amines **a.** dopamine (DA), **b.** octopamine (OA) and **c.** serotonin (5-HT) in **1.** brain and **2.** hemolymph tissue of 10-day old laboratory-maintained QL workers with differing degrees of ovary activity. 5-HT was not detected in the hemolymph so is not shown. Ovary scores range from 0-3 (see Figure 2.2), where a higher score (darker shade) relates to a higher degree of ovary activity. N = 6, 10, 5 and 7 for ovary scores 0, 1, 2 and 3 respectively. Differences in biogenic amine levels between ovary development stages were analysed using GLMMs with *post-hoc* testing carried out using estimated marginal means. Significant pairwise comparisons to the level of $p < 0.05$ and bellow are denoted by boxplots not sharing a letter. Non-significant pairwise comparisons are denoted by 'n.s'. For full table of pairwise comparisons for brain dopamine, see Table 3.2

I also hypothesised that dopamine mediates QMP's repression of ovary activity by acting as a circulatory hormone and interacting directly with ovarian tissue. Dopamine was detected in both hemolymph and ovary samples, indicating a possible role in signalling to peripheral tissues. However, dopamine levels in these tissues were not significantly altered by QMP presence (Figure 3.4). In workers from the hive, no effect of queen status, age, or their interaction on ovary dopamine levels was found (GLMM; Queen status: $\chi^2 = 3.52$, df = 2,69, p = 0.17; Age: $\chi^2 = 1.38$, df = 2,69, p = 0.50; Interaction: $\chi^2 = 0.01$, df = 1,70, p = 0.93). In parallel with brain dopamine levels, Hemolymph dopamine

levels were also reduced in QR workers (Figure 3.4), though these reductions in the hemolymph were not statistically significant (LMM; Queen status: $\chi^2 = 3.49$, df = 2,81, p = 0.17). Hemolymph dopamine levels did significantly decrease with age, though there were no significant interaction effects between age and colony queen status (Age: $\chi^2 = 9.20$, df = 2,81, $p < 0.05$; Interaction: $\chi^2 = 0.17$, df = 1,82, p = 0.68). Similarly, workers maintained in the laboratory showed no difference in hemolymph dopamine titres between QL and QR microcolonies (Figure 3.5; MWU; W = 363, df = 56, p = 0.3752), suggesting that QMP does not affect titres in the hemolymph as it does in the brain. Additionally, hemolymph dopamine levels did not differ significantly between QL workers with different degrees of ovary development (Figure 3.6; GLMM; $\chi^2 = 0.88$, df = 3,23, p = 0.83), providing no evidence that ovary activation is initiated by changes in hemolymph dopamine titres.

3.3.5 Octopamine is modulated by QMP

Evidence to support a function of octopamine as a circulatory hormone acting directly on ovarian tissue was mixed. Although not detected in the ovary itself, octopamine was present in the hemolymph, and titres were significantly reduced in QR relative to QL workers sampled from the hive (Figure 3.4; GLMM; $\chi^2 = 20.77$, df = 2,79, $p < 0.001$). *Post-hoc* testing showed differences in brain octopamine to be statistically significant only in 10-day old workers (Z = -2.36, $p < 0.01$; For full table of all pairwise comparisons see Appendix B, Table B.1). No effect of QMP on hemolymph octopamine levels was observed in the laboratory (Figure 3.5; MWU; W = 430, df = 56, p-value = 0.89), and there was no correlation between hemolymph octopamine and ovary score in workers from QL microcolonies (Figure 3.6; GLMM; $\chi^2 = 1.63$, df = 3,23, p = 0.65). In the hive, hemolymph octopamine was further affected by age, with reduced levels at 21 relative to 10 days in both QL and QR hives (Figure 3.4; GLMM; $\chi^2 = 19.95$, df = 2,79, $p < 0.001$). No interaction effect between age and colony queen status was observed (GLMM; $\chi^2 = 3.64$, df = 1,80, p = 0.06).

Multiple lines of evidence were found supporting a link between brain octopamine and QMP's repression of ovary activity. In the hive, brain octopamine levels were unaffected by age and its interaction with colony queen status (Figure 3.4; GLMM; Age: $\chi^2 = 5.62$, df = 2,82, p = 0.07; Interaction: $\chi^2 = 2.20$, df = 1,83, p = 0.14), but were significantly reduced in QR relative to QL workers (GLMM; $\chi^2 = 22.45$, df = 2,82, p <0.001). Brain octopamine levels were also depressed in workers from QR laboratory microcolonies (Figure 3.5; MWU; W = 636.5, df = 56, p < 0.001), indicating that this is in direct response to QMP exposure. Similarly to dopamine, in QL microcolonies increasing brain octopamine levels were associated with increasing degrees of ovary activity (Figure 3.6), though this was not statistically significant (GLMM; $\chi^2 = 4.13$, df = 3, 24, p = 0.25). Nonetheless, this points to a tentative role of octopamine in the control of honey bee worker reproduction.

3.4 Discussion

Biogenic amines are a highly conserved class of molecules which function as neurotransmitters, neuromodulators and neurohormones (Evans, 1980). They have diverse roles in the co-ordination of division of labour in eusocial insect societies (Reviewed by Sasaki & Harano, 2010), with dopamine in particular implicated in the mediation of reproductive constraint in a range of eusocial species (Sasaki & Harano, 2010). In honey bees, QMP's modulation of dopamine in the brain is thought to underly the inhibition of worker reproduction (Beggs *et al*., 2007; Harris & Woodring, 1995; Sasaki & Nagao, 2001), though there has been a distinct shortage of studies linking these changes in the brain with the modulation of peripheral ovarian tissue. In this chapter, the hypothesis that biogenic amines have additional roles as QMP-responsive circulatory hormones and interact directly with ovarian tissue was empirically tested. In parallel to this, the effects of QMP on these biogenic amines in the brain were characterised, highlighting for the first time a possible role of octopamine acting alongside dopamine in the regulation of worker reproduction.

Brain octopamine was consistently depressed in QR workers both in the hive and the laboratory, implicating this molecule as a key mediator of QMP's effects. Brain

octopamine titres have not previously been found to be altered by QMP (Harris & Woodring, 1995). However, the HPLC methodologies used for biogenic amine quantification differed considerably with those used in this study, where the derivatization of compounds in combination with fluorescent detection enabled highly sensitive determination of octopamine titres which may have revealed differences not previously seen.

QMP's depression of brain octopamine suggests an involvement of this molecule in the mediation of worker behavioural or physiological responses to the queen and QMP. The additional finding that brain octopamine levels positively correlated with ovary activity in QL workers (though not statistically significantly) points to a speculative role of octopamine as a positive regulator of ovary development. Octopamine is involved in the regulation of reproduction in a range of insects; For instance, egg laying is stimulated by the injection of octopamine in the rice leaf bug, *Trigonotylus caelestialium* (Yamane, 2013) and the diamondback moth, *Plutella xylostella* (Li *et al*., 2020). In *D. melanogaster,* octopamine regulates ovulation and the muscle contractions involved in egg-laying (Meiselman *et al*., 2018; White *et al*., 2021). It is therefore not beyond reason to speculate that a conserved gonadotropic function of octopamine may also exist in honey bees.

The effects of octopamine in honey bees have previously been restricted to the mediation of behavioural maturation and division of labour. The elevation of brain octopamine levels with age is a driver of temporal polyethism by mediating the behavioural switch to foraging in older workers (Wagener-Hulme *et al*., 1999). Treatment of young workers with octopamine has also been demonstrated to induce precocious foraging (Schulz *et al*., 2002; Schulz & Robinson, 2001). The observed reduction in hemolymph octopamine between 10 to 21 days is contrary to this, and this discrepancy with previous studies is attributed to seasonal effects. Octopamine levels, along with foraging rates, decline into the Autumn (Harris & Woodring, 1992). As sampling from the hive occurred in September, overall seasonal reductions in foraging may explain the observed decrease in octopamine levels between timepoints. This may

limit the validity of between-timepoint comparisons, but is unlikely to confound comparisons of biogenic amine levels between QR and QL workers within each timepoint, as differences arising from exposure to queen pheromones should be unaffected.

In keeping with previous studies, brain dopamine levels correlated positively with ovary activity (Harris & Woodring, 1995; Sasaki & Nagao, 2001) and were reduced in QR relative to QL workers sampled from the hive (Beggs *et al*., 2007), adding to existing evidence that depression of brain dopamine by QMP may enforce worker reproductive constraint. However, brain dopamine levels were not reduced by QMP in workers from laboratory-maintained microcolonies despite its repression of ovary activity. This may indicate that dopamine, and other biogenic amines, are not the sole regulators of QMP's effects on reproduction. The reduced brain dopamine levels seen in QR workers from the hive may have arisen from an additional component not present in the laboratory, such as brood pheromone (Mohammedi *et al*., 1998; Pankiw & Garza, 2007). The presence of additional regulatory components in the hive would explain the tighter inhibition of reproductive constraint seen in the QR hive compared with QR laboratory microcolonies, where in the laboratory we see approximately 11% ovary activation even in the presence of synthetic QMP (Figure 3.3). However, while multiple mechanisms of reproductive constraint are probable, this is not a likely explanation for the lack of dopamine depression in QR laboratory microcolonies. Notably, the QMP component homovanillyl alcohol (HVA), which has been directly implicated in the depression of brain dopamine levels (Beggs *et al*., 2007), was present in the synthetic QMP blend used in laboratory trials.

The observed depression of brain dopamine levels in QR workers from the hive but not in the laboratory could be explained by the different rates of ovary development in each setting. Laboratory microcolonies received a high-protein diet specifically formulated to permit ovary activation (Duncan *et al*., 2016), which may substantially accelerate ovary development compared with the hive. In the hive, workers sampled showed no signs of ovary activation until day 21, at which mature oocytes were present in only 12% of

workers. Meanwhile, in laboratory microcolonies 42% of QL workers possessed fully mature oocytes within 10 days. Brain dopamine levels were found to positively correlate with ovary development during the initial stages of ovary activation (scores 0-2; Figure 2.2) followed by a drop in workers possessing fully mature oocytes, implying an involvement of dopamine in the initial stages of oogenesis. The lack of difference in dopamine levels in the laboratory may therefore be reflective of the higher proportion of workers at later stages of ovary maturity than was seen in the hive at either time point.

Multiple lines of evidence support a role for dopamine in regulating the early processes of oogenesis. Previous work has also found reduced brain dopamine levels in QR relative to QL workers in the hive which were not replicated in 6-12 day old workers in the laboratory (Harris & Woodring, 1995). Where depression of brain dopamine by QMP has been observed in the laboratory previously, workers were just two-days old (Beggs *et al*., 2007). At two-days old laboratory workers are at the beginning of the pre-vitellogenic stage, whereas by 10-days old they are reaching peak vitellogenic stage in which mature oocytes are formed (Koudjil & Doumandji, 2008). The mechanism that maintains worker sterility within the ovary tissue acts on the earliest stages of development; In the presence of QMP, Notch signalling acts in the germarium, the region of the ovary where oocytes are specified, to actively inhibit oogenesis (Figure 1.4; Duncan *et al*., 2016). Further, the expression of the dopamine receptor *AmDop1* in worker ovaries is localized to the germarium (Duncan, unpublished data; see Appendix F, Figure F.1) and is up-regulated in two-day old QL workers (Vergoz *et al*., 2012). This indicates a potential link between dopamine and the initiation of early stages of oogenesis via the inhibition of Notch signalling, though further study is required to establish whether dopamine receptor activity can modulate Notch signalling (refer section 7.3.3, General Discussion). An investigation into the temporal dynamics of brain dopamine levels following the loss of a queen and how these correspond to ovary developmental stage is also needed to establish the temporal dynamics of changes in dopamine levels preceding ovary activation in both the laboratory and in the hive.

Links between brain biogenic amine titres and the regulation of peripheral tissues are still somewhat uncertain. Evidence was found in support of the idea that dopamine and octopamine have additional roles as circulating neurohormones. As well as being present in the brain, both dopamine and octopamine were detected in the hemolymph, hence direct interaction of these biogenic amines with the AmDop1, AmDop3 and AmOA1 receptors present in the ovarian tissue is feasible (Vergoz *et al*., 2012). There was also evidence to suggest that queen pheromones are capable of modulating these biogenic amines the hemolymph. Hemolymph titres of both dopamine and octopamine were reduced in workers from QR relative to QL hives, though this was statistically significant only for octopamine (Figure 3.4). The reductions in hemolymph octopamine could directly regulate the repression of ovary activity through reduced activation of the ovarian AmOA1 receptor, and may be a key mechanism underlying reproductive constraint. However, the suppression of hemolymph octopamine seen in the QR hive was not observed in QR laboratory microcolonies receiving synthetic QMP (Figure 3.5), raising the same questions as discussed above for brain dopamine: it this due to the temporal dynamics of biogenic amine modulation, or the additional queen pheromones not present in the laboratory?

Regardless of the differences observed between workers from the hive and laboratory microcolonies, the suppression of hemolymph octopamine in QR hives could be an important regulator of reproductive constraint. Ovarian biogenic amine receptor expression is dynamic and is also influenced by QMP presence, which may serve as an additional axis for the mediation of ovary development. AmOA1 expression in the ovary is reduced at later developmental stages (Vergoz *et al*., 2012), perhaps reflecting a loss of capacity to respond to changes in hemolymph octopamine that signal the queen's presence once ovaries are fully mature. Although hemolymph dopamine titres were not reduced by QMP to a statistically significant degree, ovarian responses to dopamine may also still be affected through the rapid alteration in expression levels of the antagonistic dopamine receptors *AmDop1* and *AmDop3* in the ovary in response to the absence of QMP (Vergoz *et al*., 2012).

If dopamine and octopamine in the hemolymph function as circulating neurohormones mediating ovary development, the relationship between brain biogenic amines and reproduction becomes less clear. In particular, it is unclear whether QMP, and possibly other queen or brood pheromones, co-modulate biogenic amines in the brain and hemolymph through a shared mechanism, or whether these are distinct processes. The source of biogenic amines in the hemolymph, and hence how they may be regulated, is uncertain; For instance, they could be secreted from peripheral tissues, secreted from the brain itself, or synthesized in the hemocytes as in the moth, *Chilo suppressalis* (Wu *et al*., 2015). In addition, dopamine and octopamine in the brain could regulate reproduction via additional mechanisms that have not been considered here, such as through regulation of the wider neuroendocrine signalling network (i.e. Insulin/20E pathways) or via neuronal signalling. These ideas are discussed in greater depth in section 7.3.2, General Discussion.

In this chapter, further supporting evidence for a regulatory role of dopamine in QMP's inhibition of worker reproduction is demonstrated, with a hypothesised role early in the initiation of oogenesis. Regulation of octopamine, whether by QMP or by other queen or brood pheromone components, is additionally proposed as a mediator of ovary development. The presence of these biogenic amines in the hemolymph, and the responsiveness of octopamine in particular to the presence of a queen, indicates that direct effects on ovary development via interaction with the ovary tissue is possible. Further study is required to determine the relationship between brain and hemolymph biogenic amine titres; In particular, whether these represent part of the same mechanism regulating ovary development, or distinct regulatory pathways.

Chapter 4

Testing the causal relationship between dopamine levels and ovary development

4.1 Introduction

Biogenic amines, in particular dopamine, have been proposed as mediators of QMP's induction of sterility in honey bee workers (Section 1.5.3; Also reviewed by Sasaki & Harano, 2010; Sasaki *et al*., 2021). In Chapter three, additional evidence was found to support a role for dopamine, and possibly octopamine, in regulating reproductive constraint. Most notably, brain dopamine levels were shown to be reduced in workers from queenright (QR) relative to queenless (QL) hives (consistent with previous studies; Beggs *et al*., 2007; Harris & Woodring, 1995; Sasaki & Nagao, 2001), suggesting that QMP, and possibly other queen pheromones, are capable of modulating dopamine levels in workers. In addition, it was demonstrated that brain dopamine levels positively correlate with the initial stages of ovary development in QL workers, consistent with the positive relationship between ovary activity and dopamine levels reported by Sasaki & Nagao (2001). While this adds to a large body of circumstantial support for a role of dopamine in mediating QMP's inhibition of reproduction, it could equally be concluded from the findings of Chapter three and of previous studies that engaging in ovarian development in the absence of the queen simply triggers a rise in dopamine titres in the brain. As such, it is integral to establish the causal relationship between dopamine levels and ovary activity to progress the idea that QMP's modulation of dopamine is a driver of sterility in workers.

In *Drosophila virillis,* dopamine's function as a gonadotropin has been well established. In this species, dopamine positively regulates oogenesis predominantly via regulation of the gonadotropin juvenile hormone (JH) (Gruntenko *et al*., 2005, 2007). A shortage of empirical studies from other species means that the extent to which the neuroendocrine signalling network in *Drosophila* species is representative of all insects is unclear. For

instance, the gonadotropic function of JH has been lost in some advanced eusocial species, including the honey bee (Rodrigues & Flatt, 2016), and it is not completely clear how the neuroendocrine network has been rewired to accommodate this loss of function. Such differences in neuroendocrine signalling between insect species emphasise the need for establishing the specific roles of hormones and signalling molecules on a species-by-species basis (Reviewed by Knapp, Norman *et al*., 2022, in press).

In eusocial insects, evidence implicating dopamine as a regulator of ovary development is predominantly correlative. For instance, there is a positive association between brain dopamine levels and ovarian development in paper wasp workers (Sasaki *et al*., 2007; Yoshimura *et al*., 2021), bumble bees (Bloch *et al*., 2000; Sasaki *et al*., 2017), and reproductive females (gamergates) in derived queenless ant species (Okada *et al*., 2015; Penick *et al*., 2014). However, some manipulative studies have begun to address the causal relationship between dopamine levels and ovary activity. For example, supplementary dopamine feeding enhanced reproductive development in workers of the paper wasp *Polistes chinesis* (Sasaki *et al*., 2009) and derived queenless *Diacamma* ants (Okada *et al*., 2015). A gonadotropic function of dopamine has been most convincingly shown in the fire ant *Solenopsis invicta,* where the inhibition of dopamine biosynthesis through treatment with the dopamine synthesis inhibitor 3-iodo-L-tyrosine led to reduced oocyte numbers (Boulay *et al*., 2001). Normal reproductive function was restored with the application of the dopamine precursor L-Dopa (Boulay *et al*., 2001), suggesting that dopamine is required for ovarian development.

Circumstantial evidence for a gonadotropic function of dopamine in honey bees has been discussed in previous sections (i.e. Section 1.5.3, General Introduction) and also demonstrated in Chapter three, where brain dopamine levels positively correlated with ovary activity in QL workers (consistent with Sasaki & Nagao, 2001). It has also been well established from previous work that QMP depresses brain dopamine levels in workers (Beggs *et al*., 2007; Harris & Woodring, 1995; Sasaki & Nagao, 2001; Chapter three), with the QMP component HVA shown to be at least partially responsible for this depression

(Beggs & Mercer, 2009). From this, along with the gonadotropic function of dopamine in other related eusocial Hymenopterans (Boulay *et al*., 2001; Okada *et al*., 2015; Sasaki *et al*., 2009), QMP's depression of brain dopamine levels has been assumed to be a cause of ovary inhibition in honey bee workers (i.e. Sasaki and Harano, 2010; Sasaki *et al*., 2021). However, empirical support for a gonadotropic effect of dopamine in honey bees rests largely on just one highly cited study, in which dopamine supplementation was found to enhance ovary activation rates in queenless (QL) honey bee workers (Dombroski *et al*., 2003). Despite a further two decades of research, the assumed causal effect of dopamine on ovarian development in honey bees still rests solely on this study, with no published replications of these findings to my knowledge.

In addition, Dombroski *et al*., (2003)'s finding that supplementing QL workers with dopamine further promotes ovary activity suggests that dopamine has a positive effect on ovary development in workers already free from QMP's inhibitory effects. However, it does not test the hypothesis that QMP's depression of dopamine is responsible for the repression of ovary development in QR workers. For instance, if QMP's depression of dopamine is a mechanism by which reproductive constraint is enforced, I hypothesise that restoring the dopamine levels of workers to 'QL' levels through dietary dopamine supplementation should at least partially overcome QMP's inhibitory effects. Similarly, if dopamine is required for ovarian development, reducing the dopamine levels of workers through treatment with a dopamine synthesis inhibitor (i.e. as in Boulay *et al*., 2001) should stunt the ovary development of QL workers.

This chapter aims to validate the assumed gonadotropic effect of dopamine in honey bee workers proposed in chapter three by seeking to replicate the results reported by Dombroski *et al*., (2003). Initial dopamine supplementation regimes are carried out as by Dombroski *et al*., (2003), with further experiments testing the effect of both dopamine and its precursor L-Dopa on ovarian development at a wider range of doses. In addition, the hypothesis that QMP's depression of brain dopamine inhibits ovary activation in QR workers is addressed by extending dopamine supplementation regimes to also include workers exposed to QMP. The hypothesis that dopamine is required for

ovary activation was also tested by observing the effects of inhibiting dopamine biosynthesis on ovary development. This extensive series of manipulative experiments provides a broader framework of empirical evidence from which the causal relationship between dopamine levels and ovary activity in honey bee workers, and hence the mechanism behind QMP's induction of sterility, can be inferred.

4.2 Methods

4.2.1 Laboratory microcolony set-up

A. mellifera hives were maintained as described previously (Section 2.1.1). Laboratory microcolonies containing 80-100 workers were setup in cages as in Section 2.1.1 and underwent a variety of dopamine supplantation and inhibition treatments to elucidate the causal relationship between dopamine levels and ovary activity. The ovaries of all remaining workers from each microcolony were dissected, imaged and scored as described in Section 2.2 after 10 days.

4.2.2 Dopamine supplementation treatments

Initial dopamine supplementation experiments aimed to replicate the results of Dombroski *et al*., (2003), in which dietary dopamine increased ovary activation in QL workers. QL laboratory microcolonies containing 100 workers were setup between June-July 2018 with three independent replicates of each treatment cage. Dietary dopamine was administered following the methodology of Dombroski *et al*., (2003) by adding 0.01 mg dopamine/g food offered to DA+ cages, but not DA- (control) cages. To additionally assess whether dietary dopamine can overcome QMP's repression of ovary activity, dopamine treatments were tested in both the presence (QMP+) and absence (QMP-) of synthetic QMP. QMP treatments were administered following the methodology described in section 2.1.2.

In subsequent attempts to replicate the phenotype of Dombroski *et al*., (2003), dietary dopamine was also tested at the increased dosages of 0.1, 0.5, 1 and 2 mg/g food in

cages of 100 workers maintained in the absence of QMP only. Cages were setup between August-October 2018 with three independent replicates of each treatment cage.

4.2.3 L-Dopa supplementation treatments

In further attempts to replicate the results of Dombroski *et al*., (2003), treatment with the dopamine precursor L-dopa (Figure 4.1) was trialled as an alternative means of dopamine supplementation. L-Dopa is commonly used as a drug in humans to restore dopamine levels in the brain as a treatment for Parkinson's disease, and is favoured over treating with dopamine directly as it crosses the blood-brain-barrier more effectively (Haddad *et al*., 2018). It was hence hypothesised that L-Dopa treatment may also lead to more effective elevation of dopamine levels in honey bee worker brains. Effects of the dopamine precursor L-dopa on ovary activation were tested in the absence of QMP only. Two experimental cage replicates were set up between July-August 2019, with a final third replicate carried out in August 2020. Microcolonies contained 80 workers due to constraints on ovary dissection capacity at the end of the experiment. As L-dopa has

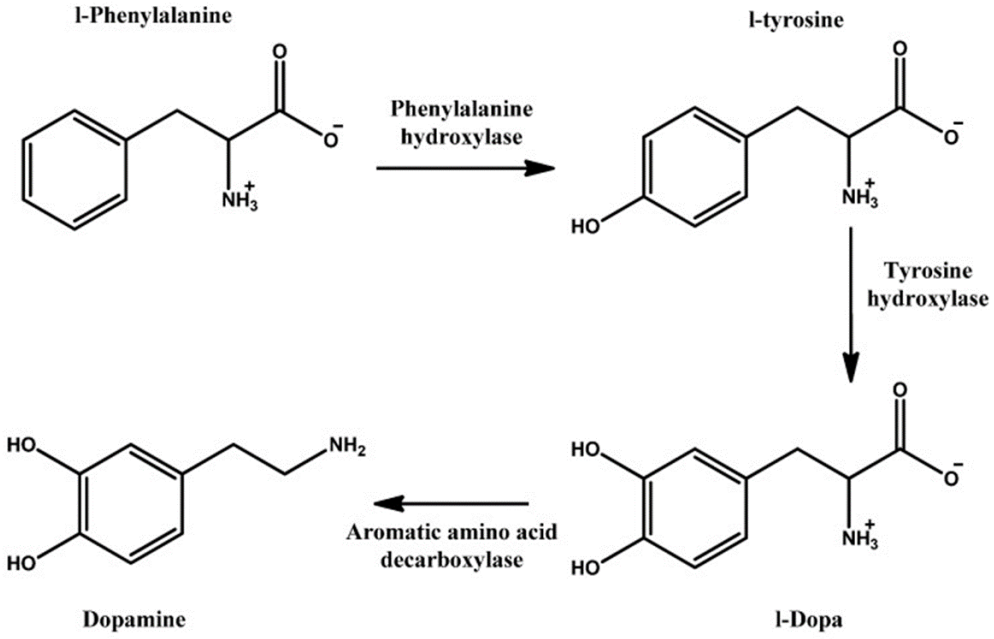

Figure 4.1 Schematic of the dopamine biosynthesis pathway Tyrosine hydroxylase converts L-Tyrosine to the dopamine precursor L-Dopa. Inhibiting the action of tyrosine hydroxylase using the inhibitor 3-iodo-L-tyrosine therefore leads to reduced dopamine biosynthesis. Figure sourced from Haddad *et al*., (2018) (open access)

lower water solubility than dopamine (0.99 mg/mL as opposed to 18.96 mg/mL), it was not possible to spike the food with concentrated solution as was carried out for dopamine treatments without the food mixture becoming too runny. L-dopa was instead administered by spiking water solutions offered to cages at concentrations of 0.01, 0.1 and 0.5mg/mL, while controls received just water. Every day L-dopa solutions were replaced with pre-made solutions stored at -20°C. To assess the impact of L-dopa supplementation on biogenic amine levels, brains were also collected from a subset of five randomly selected bees per cage on day 10 for later quantification of dopamine. Octopamine and serotonin levels were also quantified in case of possible effects of L-Dopa supplementation on non-target biogenic amines. Brain samples were collected, prepared and analysed using HPLC-FLD as described in section 2.3.

4.2.4 Dopamine inhibition treatments

To test whether a reduction in dopamine levels alone could mirror QMP's inhibitory effects on ovary activity, dopamine levels were experimentally reduced through administration of the dopamine synthesis inhibitor 3-iodo-L-tyrosine (iodotyrosine). Iodotyrosine inhibits tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of the dopamine precursor L-Dopa (Figure 4.1), and has been shown to reduce dopamine levels in *D. melanogaster* (Neckameyer, 1996) and fire ants (Boulay *et al*., 2001). Iodotyrosine was supplied to bees by spiking their water solutions at concentrations of 0.01, 0.1 and 0.5 mg/mL, while controls received just water (Similar to administration methodology used by Boulay *et al*., 2001). As above, iodotyrosine solutions were replaced with frozen pre-made solutions each day. To determine whether dopamine inhibition could exacerbate QMP's repression of ovary activity, two experimental cages were setup for each iodotyrosine treatment, one maintained in the presence (QMP+) and one in the absence of synthetic QMP (QMP-). QMP treatments were administered following the methodology described in Section 2.1.2. Three independent replicates for all eight treatment combinations were carried out between August-September 2019. Experimental cages again each contained 80 workers in line with ovary dissection capabilities.

4.2.5 Data analysis

Data analysis was carried out in R. Details of packages used for analysis are found in section 2.4. In all experiments in which a range of doses of each treatment solution were used (i.e. some dopamine experiments, L-Dopa experiments, iodotyrosine experiments), dose was considered an ordinal variable in all analyses, as the number of doses tested was insufficient to be considered a continual variable.

Food intake was compared across treatment groups for each experiment by fitting linear mixed effects models (LMMs) with mean food intake per bee per day as the response variable. Where treatments were administered in water solutions (L-dopa and iodotyrosine), additional analyses with water intake per bee per day as the response variable were also performed. In initial dopamine supplementation experiments in the presence and absence of QMP, dopamine, QMP presence and their interaction were included as fixed effects. In subsequent experiments testing dopamine and L-dopa at a range of doses in the absence of QMP only, dose was included as the sole fixed effect. For dopamine inhibition experiments fixed effects were iodotyrosine dose, QMP presence and their interaction. To account for repeated measures, day was included as a random effect and nested within replicate for all models. Visual examination of all model residual plots revealed no obvious deviations from normality or homoscedasticity. Statistical significance of fixed effects was determined by comparing the likelihood ratio of the maximal model to that of the null model, or model without the fixed effect of interest. If fixed effects were statistically significant, *post-hoc* testing was carried out using estimated marginal means to determine significance of all pairwise treatment comparisons. P-values were Tukey adjusted to control for multiple testing.

In each experiment survival distributions were compared between treatment groups using Cox proportional hazards (CPH) models with mixed effects. Analysis of initial dopamine supplementation experiments involving QMP included dopamine, QMP presence and their interaction as fixed effects. In subsequent dopamine and L-dopa dosage experiments dopamine or L-dopa dose was the sole fixed effect. Survival rates

in dopamine inhibition experiments were analysed with iodotyrosine dose, QMP and their interaction as fixed effects. In all models replicate was included as a random effect and the assumption of proportional hazards was verified by visual examination of the correlation of scaled Schoenfield residuals with time to test for independence. In each analysis CPH models were used to predict Hazard Ratios (HR) and confidence intervals (CI) for fixed effects. HRs are presented as HR (±95% CI). Where fixed effects were statistically significant, *post-hoc* testing was carried out using estimated marginal means to determine the significance of pairwise treatment comparisons. P-values were Tukey adjusted to control for multiple testing.

To assess the effect of different dopamine and QMP treatments on ovary activity, cumulative link mixed effects models (CLMMs) were used with ovary score as an ordinal response variable. In initial dopamine supplementation experiments in the presence and absence of QMP, CLMMs were fitted with dopamine, QMP presence and their interaction as fixed effects. In subsequent experiments testing dopamine and L-dopa supplementation at a range of doses in the absence of QMP only, dose was considered an ordinal variable and modelled as the sole fixed effect. For dopamine inhibition experiments fixed effects were iodotyrosine dose, QMP and their interaction. Replicate was included as a random effect for all models. In each analyses statistical significance of fixed effects was determined by comparing the likelihood ratio of the maximal model to that of the null model, or model without the fixed effect of interest. Where fixed effects were statistically significant, *post-hoc* testing was carried out by computing least-squares means to determine the significance of pairwise comparisons. P-values were Tukey adjusted to control for multiple testing.

Brain levels of dopamine, octopamine and serotonin following different L-dopa treatment regimes were each assessed by fitting GLMMs with a gamma distribution and an inverse link function. L-dopa dose was included as the fixed effect and replicate and sample preparation block were included as random effects. GLMMs were deemed more

appropriate for the data than LMMs, as LMMs which were initially fitted and were found not to meet the assumptions of normality or homoscedasticity of model residuals. Visual examination of GLMM model residual plots revealed no obvious deviations from normality or homoscedasticity. Statistical significance of L-dopa treatment on each biogenic amine was assessed by comparing the likelihood ratio of the full model to that of the null model containing no fixed effects.

4.3 Results

4.3.1 Dopamine has no effect on ovary activity in the presence or absence of QMP

Initial dopamine supplementation experiments saw dopamine administered to workers based on the methodology of Dombroski *et al*., (2003) in an attempt to replicate these findings and additionally test dopamine's effects in the presence of QMP. Survival rates and food consumption levels were compared across treatments to examine whether dopamine supplementation regimes caused non-target effects on nutritional status or mortality (Figure 4.2). Dietary dopamine treatment did not significantly compromise survival rates (CPH: HR = 0.96 ± 0.17 SE, z = -0.28, p = 0.78), nor did QMP exposure (CPH: HR = 1.16 ± 0.16, z = 0.91, p = 0.36) or the combination of the two (CPH: HR = 1.38 ± 0.22, z = 1.44, p = 0.15). Food consumption rates were also consistent across treatments, with bees from dopamine-treated cages consuming an average of 0.13 µg dopamine per bee per day. Food intake did not differ significantly with dopamine, QMP exposure or their

Post-hoc pairwise comparisons of ovary scores between dopamine supplementation treatments (+/- QMP)

Contrast	Estimate	SE	df	Z-ratio	P-value
QMP- DA- vs QMP+ DA-	-1.268	0.213	Inf	-5.965	<0.0001***
QMP- DA- vs QMP- DA+	0.185	0.192	Inf	0.965	0.7694
QMP- DA- vs QMP+ DA+	-0.920	0.210	Inf	-4.379	0.0001***
QMP+ DA- vs QMP- DA+	1.454	0.210	Inf	6.918	<0.0001***
QMP+ DA- vs QMP+ DA+	0.348	0.221	Inf	1.580	0.3903
QMP- DA+ vs QMP+ DA+	-1.105	0.208	Inf	-5.321	<0.0001***

Table 4.1 *Post-hoc* pairwise comparisons of ovary scores between all QMP/DA treatment combinations. *Post-hoc* comparisons were computed using least squares means on the maximal CLMM.

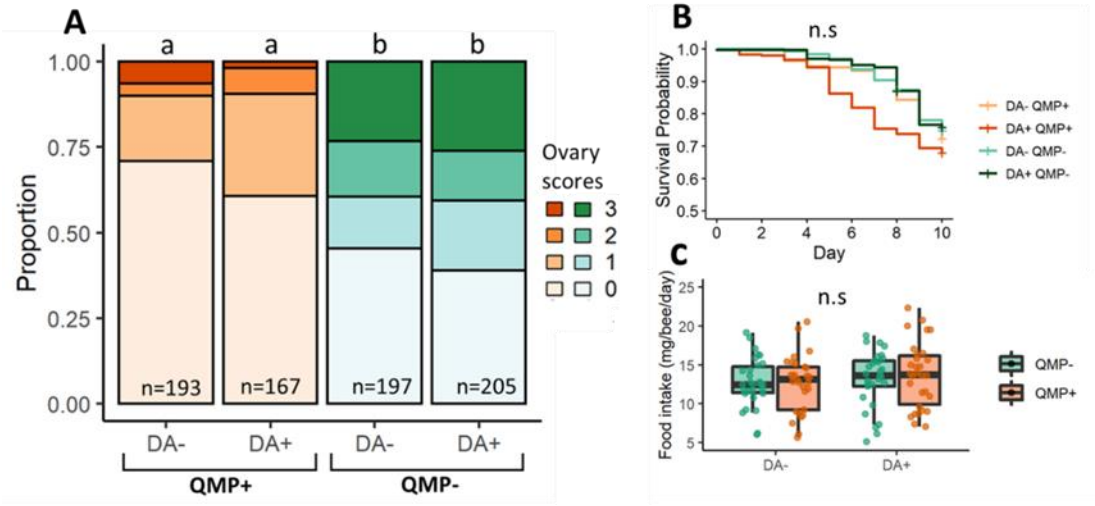

Figure 4.2 Dopamine supplementation in the presence and absence of QMP **A.** Ovary activity is reduced in bees exposed to QMP (orange bars, QMP+) relative to unexposed bees (green bars, QMP-), while dopamine supplementation at 10 µg/g food (DA+ vs DA-) has no effect. Ovary activity is shown as the proportion of bees within each treatment group with each ovary score (ranging from 0-3; Figure 2.2), where a higher score (darker shade) relates to a higher degree of development. N values for each treatment pooled across three replicates are displayed at the base of each bar. Statistical significance of *post-hoc* pairwise comparisons is denoted by bars not sharing a letter (least squares means, $p<0.05$; see Table 4.1) **B.** Survival probability distributions of cages of 100 bees over 10 days. Data shown consists of three replicates of each treatment cage pooled together. Y axis has been truncated to begin at a survival probability of 0.5. Statistical non-significance (CPH mixed effects models) is denoted by "n.s". **C.** Mean food intake per bee per day does not differ between treatment groups, as denoted by "n.s" (LMMs). Food intake was recorded daily for each cage (n = 10 days), each of which had three replicates (n = 30 for each treatment).

interaction (Figure 4.2; GLMMs: dopamine: $\chi^2 = 1.03$, d.f = 2, 117, $p = 0.60$; QMP: $\chi^2 = 1.82$, d.f = 2, 117, $p = 0.40$; Interaction: $\chi^2 = 0.37$, d.f = 1, 118, $p = 0.54$). There is therefore no evidence that dopamine supplementation treatments caused any confounding effects on mortality or nutritional status.

Assessment of ovary activity levels between QMP+ and QMP- treatments in dopamine controls (DA-) validated the efficacy of synthetic QMP as an inhibitor of ovary development (Figure 4.2). Ovary activity levels were significantly lower in QMP exposed bees (CLMM: $\chi^2 = 64.23$, d.f = 2, 759, $p < 0.001$), with the proportion of workers with active ovaries (ovary score of 2 or above; Figure 2.2) reduced from 40% to 10% in its presence in DA- controls. However, in contrast to the findings of Dombroski *et al*., (2003),

there was no evidence to support an effect of supplementary dopamine feeding on ovary activation (Figure 4.2; CLMM: χ^2 = 3.44, d.f = 2, 759, p = 0.18). Dopamine supplementation also could not overcome QMP's inhibitory effects, as *post-hoc* testing revealed there to be no significant effect of dietary dopamine on ovary activation in the presence of QMP (All pairwise *post-hoc* comparisons displayed on Figure 4.2A and Table 4.1). Further, no interaction effects between QMP and dietary dopamine on ovary activity were observed (figure 1A; CLMM: χ^2 = 0.31, d.f = 1, 760, p = 0.58).

4.3.2 Dopamine and L-Dopa do not enhance ovary activity in QL workers at any dose

Dopamine and its precursor L-dopa were administered to bees in the absence of QMP at a range of increased doses to assess whether effects on ovary activity are dose-dependent. Dopamine treatment at doses of 0.1-2mg/g food had no significant effect on

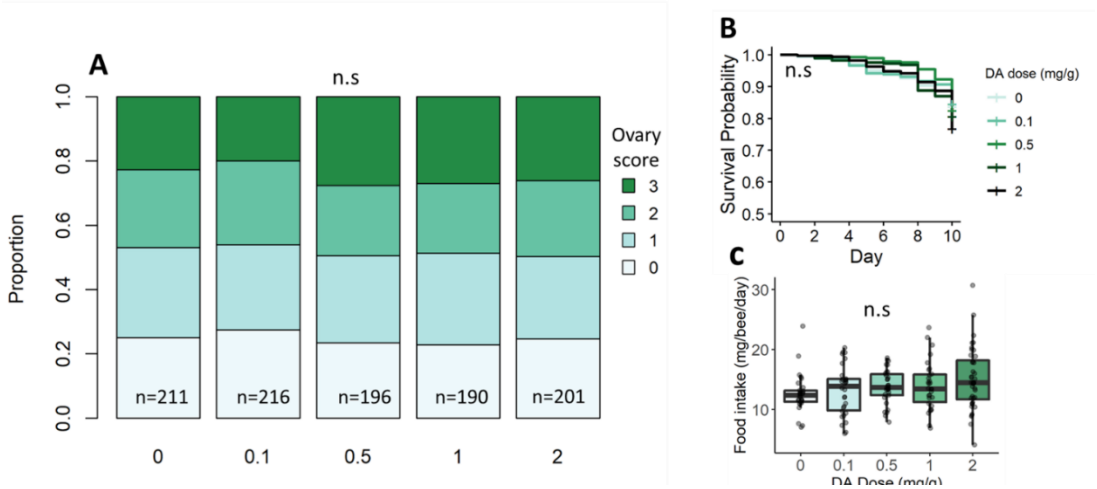

Figure 4.3 Effects of dopamine supplementation at a range of doses A. Dopamine supplementation does not enhance ovary activity of QL bees (housed without QMP) at any dose tested. Ovary activity is shown as the proportion of bees within each treatment group with each ovary score (ranging from 0-3; Figure 2.2), where a higher score (darker shade) related to higher degree of ovary development. N values for each treatment across three pooled replicates are displayed at the base of each bar. "n.s" denotes statistical non-significance (CLMMs). **B.** Survival probability distributions of cages of 100 bees over 10 days of dietary dopamine treatments. Data shown consists of three replicates of each treatment cage. Y axis has been truncated to begin at a survival probability of 0.5. Statistical non-significance (CPH mixed effects models) is denoted by "n.s". **C.** Mean food intake per bee per day does not differ with dietary dopamine dose, as denoted by "n.s" (LMMs). Food intake was recorded daily for each cage (n = 10 days), each of which had three replicates (n = 30 for each treatment).

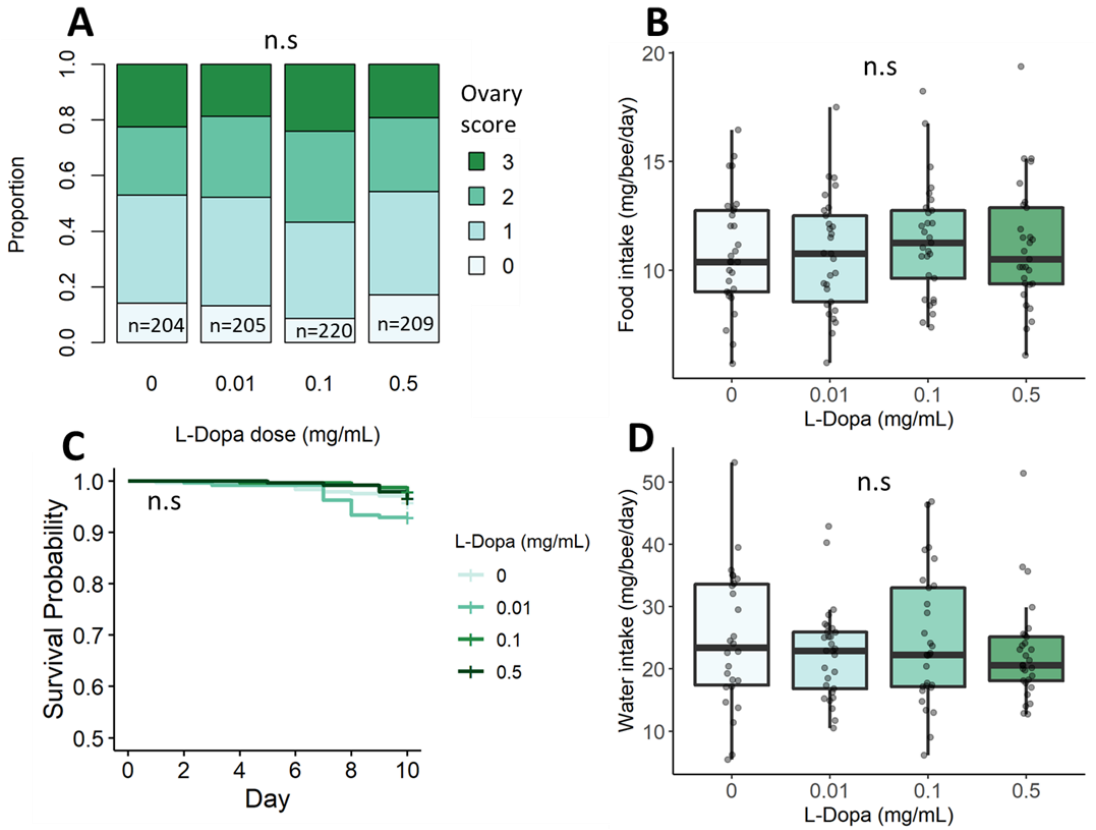

Figure 4.4 Effects of L-Dopa supplementation at a range of doses A. L-dopa supplementation does not enhance ovary activity of QL bees (housed without QMP) at any dose tested. Ovary activity is shown as the proportion of bees within each L-dopa treatment dose with each ovary score (ranging from 0-3), where a higher score (darker shade) related to higher degree of ovary development. N values for each treatment across three pooled replicates are displayed at the base of each bar. "n.s" denotes statistical non-significance (CLMMs). **B.** Mean food intake and **D.** mean water intake is not affected by L-dopa treatment, as denoted by "n.s" (LMMs). Food and water intake was recorded daily for each cage (n = 10 days), each of which had three replicates (n = 30 for each treatment). **C.** Survival probability distributions of cages of 80 bees over 10 days of different L-dopa treatments. Data shown consists of three replicates of each treatment cage. Y axis has been truncated to begin at a survival probability of 0.5. Statistical non-significance (CPH mixed effects models) is denoted by "n.s".

survival rates at the doses tested (Figure 4.3; CPH: 0.1 mg/g HR = 0.94 ± 0.21 SE, z = -0.29, p = 0.78; 0.5 mg/g HR = 1.09 ± 0.20 SE, z = 0.42, p = 0.68; 1 mg/g HR = 1.30 ± 0.20 SE, z = 1.32, p = 0.19; 2 mg/g HR = 1.42 ± 0.18 SE, z = 1.92, p = 0.05). Food consumption rates were also unaffected by dietary dopamine at these doses (Figure 4.3; LMM: $\chi 2$ = 8.60, d.f = 4, 145, p = 0.07). L-dopa treatment similarly caused no negative effects on survival rates at the doses of 0.01-0.5 mg/mL water tested (Figure 4.4; CPH: 0.01 mg/mL

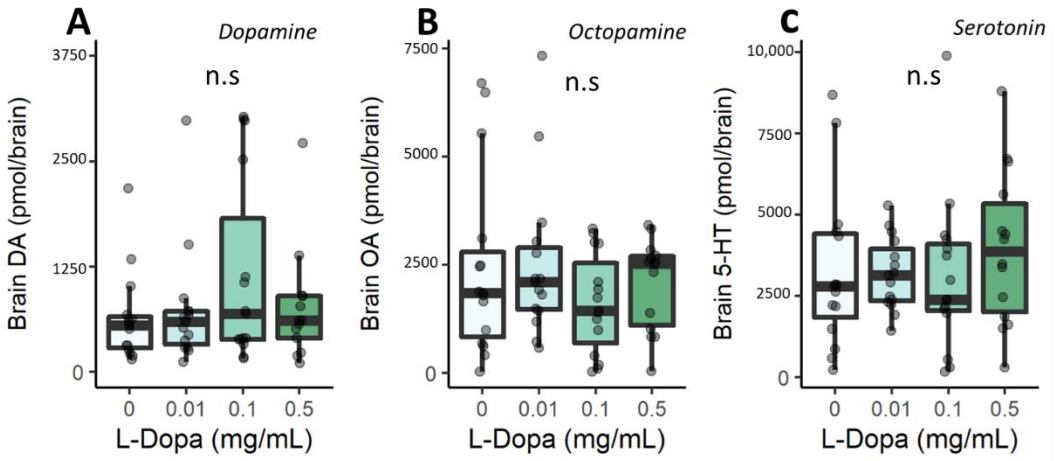

Figure 4.5 Biogenic amine levels following L-Dopa supplementation HPLC-FLD quantification of QL worker brain biogenic amines following 10-day L-dopa supplementation at a range of doses. Brains were collected from five randomly selected bees from each cage over three replicates (n=15 total for each L-dopa treatment). Levels of **A.** dopamine, **B.** octopamine and **C.** serotonin were not altered by L-dopa treatments, as denoted by "n.s" (GLMMs).

HR = 1.76 ± 0.40 SE, z = 1.41, p = 0.16; 0.1 mg/mL HR = 0.49 ± 0.55 SE, z = -1.29, p = 0.20; 0.5 mg/mL HR = 0.79 ± 0.47 SE, z = -0.49, p = 0.62). Additionally, food consumption rates did not differ with L-dopa dose (Figure 4.4; LMM: $\chi2 = 5.08$, d.f = 3, 116, p = 0.17). Water or treatment solution intake was also monitored in microcolonies receiving L-dopa treatment so that dosages of L-dopa solution received could be assessed. Solution intake was consistent across L-dopa treatment regimes, indicating that received dosages were not confounded by differential rates of consumption (Figure 4.4; LMM: $\chi2 = 2.08$, d.f = 3, 116, p = 0.56).

Despite the increased dosages of dietary dopamine tested compared with Dombroski *et al*., (2003), neither dopamine nor L-dopa supplementation enhanced ovary activity rates in the absence of QMP, conflicting with previously published data. Within the doses tested dietary dopamine had no effect on ovary activity (Figure 4.3; CLMM: $\chi2 = 1.68$, d.f = 4, 1009, p = 0.79), nor did the dopamine precursor L-dopa (Figure 4.4; CLMM: $\chi2 = 7.71$, d.f = 3, 834, p = 0.05). However, L-dopa supplementation was not found to elevate levels of dopamine or other biogenic amines (Figure 4.5). At the doses tested, L-dopa treatment was not associated with differences in brain dopamine (GLMM: $\chi2 = 3.09$, d.f

= 3, 55, p = 0.38), octopamine (GLMM: $\chi2$ = 1.44, d.f = 3, 55, p = 0.70), or serotonin (GLMM: $\chi2$ = 0.85, d.f = 3, 55, p = 0.84). The efficacy of this L-dopa supplementation method as an elevator of dopamine levels is therefore unclear.

4.3.3 Iodotyrosine represses reproduction to a degree comparable with QMP

While ovary activity could not be further increased by dopamine supplementation, treatment with the dopamine synthesis inhibitor iodotyrosine at doses of 0.1 and 0.5 mg/mL reduced ovary activation rates (Figure 4.6), implicating depressed dopamine levels as a suppressor of reproduction. Ovary activity levels were significantly lower following treatment with both QMP (CLMM; $\chi2$ = 75.49, d.f = 4, 1618, p<0.001) and iodotyrosine (CLMM; $\chi2$ = 139.65, d.f = 6, 1616, p<0.001), and there was also a significant interaction effect between the two (CLMM; $\chi2$ = 28.59, d.f = 3, 1619, p<0.001). *Post-hoc* pairwise comparisons revealed that supplementing QL workers with iodotyrosine at 0.1 mg/mL repressed ovary activity to the same extent as QMP exposure alone and significantly exceeded QMP's repression at 0.5 mg/mL (Figure 4.6). In bees already exposed to QMP, ovary activity was further repressed by iodotyrosine, though only at the highest dose of 0.5 mg/mL (Figure 4.6). A full table containing all *post-hoc* pairwise tests can be found in Appendix G (Table G.1).

Food consumption levels were consistent across all treatments, indicating that variation in ovary activity between treatment groups was not driven by differences in nutrition (Figure 4.7). Food intake was not affected by QMP (LMM; $\chi2$ = 3.06, df = 4, 235, p = 0.55), iodotyrosine (LMM; $\chi2$ = 7.50, df = 6, 233, p = 0.28), or the interaction between the two (LMM; $\chi2$ = 1.96, df = 3, 236, p = 0.58). However, it should be noted that water or solution intake was affected by treatment group (Figure 4.7), being significantly higher in QMP- relative to QMP+ bees (LMM; $\chi2$ = 38.42, df = 4, 235, p<0.001). Iodotyrosine dosage will therefore have been slightly elevated in the former, though this does not impact the interpretation of results as comparisons between iodotyrosine treatments can still be

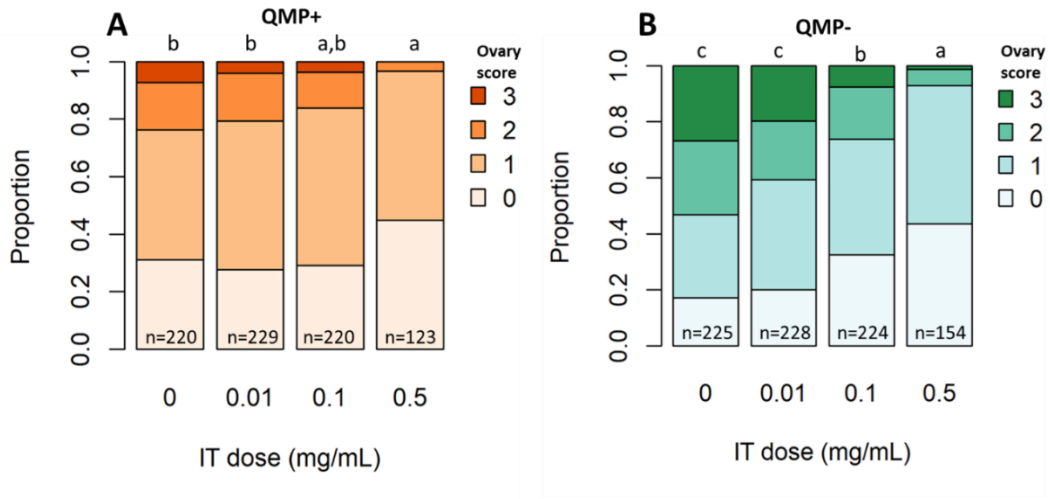

Figure 4.6 Ovary activity levels following iodotyrosine treatment Ovary activity of bees following supplementation with the dopamine synthesis inhibitor iodotyrosine at different doses in **A.** the presence of QMP (QMP+) and **B.** the absence (QMP-). Ovary activity is repressed by both QMP and iodotyrosine. Ovary activity is shown as the proportion of bees within each treatment group with each ovary score (ranging from 0-3; Figure 2.2), where a higher score (darker shade) relates to a higher degree of development. N values for each treatment pooled across three replicates are displayed at the base of each bar. Statistical significance of pairwise comparisons is denoted by bars not sharing a letter (CLMM *post-hoc* least squares means, $p<0.05$). See Appendix G, Table G.1 for a full table of all *post-hoc* pairwise comparisons.

made within QMP+ and QMP- treatment groups and between iodotyrosine controls. While model likelihood comparisons also indicated an overall significant effect of iodotyrosine supplementation on solution intake (LMM; $\chi2 = 18.05$, df = 6, 233, $p<0.01$), *post-hoc* tests revealed no significant differences between iodotyrosine doses within each QMP treatment group. These *post-hoc* pairwise comparisons are displayed in Appendix G (Table G.2).

Survival rates were also consistent across treatment groups, with the notable exception of 0.5 mg/ mL iodotyrosine for which there was a marked increase in mortality (Figure 4.7). CPH model likelihood comparisons showed survival to be significantly affected by QMP (CPH; $\chi2 = 12.76$, df = 4, $p<0.05$) and more so by iodotyrosine (CPH; $\chi2 = 359.17$, df = 6, $p<0.0001$), though their interaction was not significantly significant (CPH; $\chi2 = 6.22$, df = 3, $p = 0.10$). However, in pairwise *post-hoc* comparisons statistical significance

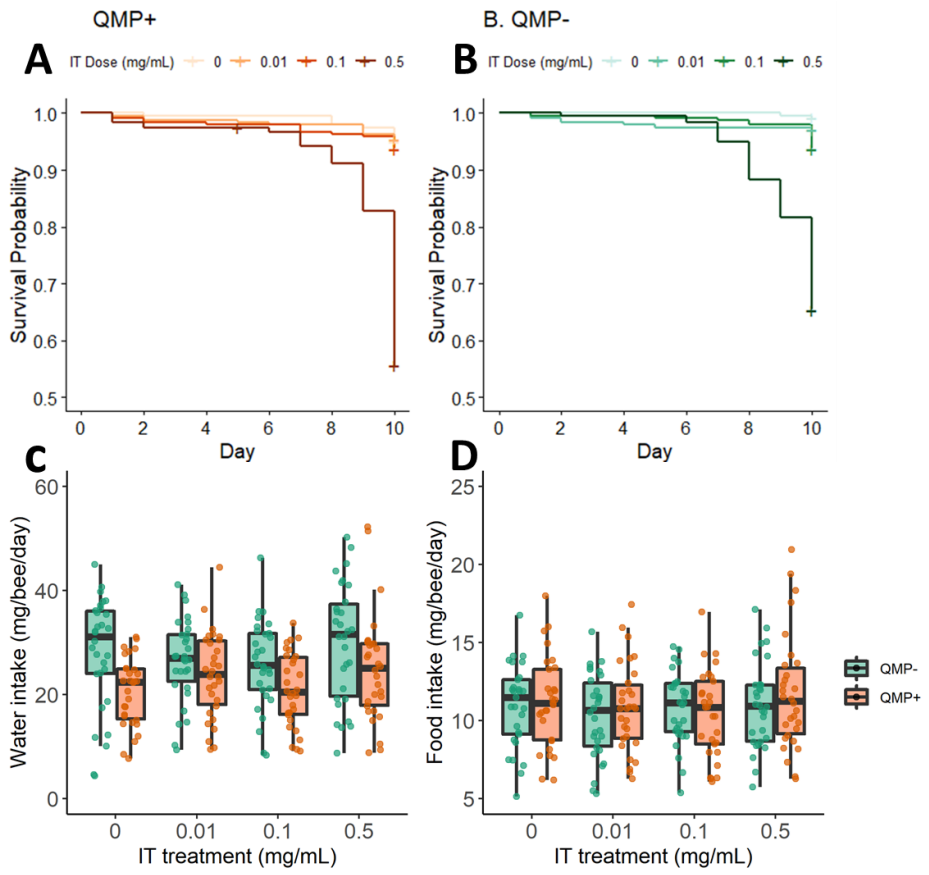

Figure 4.7 Survival and food and water intake rates of iodotyrosine treated bees
A-B Survival probability distributions of cages of 80 bees over 10 days of iodotyrosine treatments **A.** in the presence of QMP (QMP+) and **B.** in the absence (QMP-). Survival was negatively affected by iodotyrosine treatment at doses of 0.5 mg/mL only. Data shown consists of three replicates of each treatment cage. Y axis has been truncated to begin at a survival probability of 0.5. A complete list of *post-hoc* pairwise comparisons found in Appendix G, Table G.3 **C.** Mean water intake and **D**. mean food intake at different iodotyrosine supplementation doses. Food and water intake was recorded daily for each cage (n = 10 days), each of which had three replicates (n = 30 for each treatment). Food intake was consistent across treatments as denoted by "n.s" (LMMs), while water intake was reduced in QMP+ bees. Statistical significance of *post-hoc* pairwise comparisons is found in Appendix G, Table G.2

lay only between comparisons with 0.5 mg/mL treatment groups (see Appendix G, Table G.3). The reduced ovary activity seen at 0.5 mg/mL in both the presence and absence of QMP therefore cannot be separated from general toxicity of iodotyrosine at this dose. However, survival rates were not compromised by iodotyrosine supplementation at any other dose tested (Appendix G, Table G.3), indicating that the repression of QL worker

reproduction by iodotyrosine at 0.1 mg/mL was due to the inhibition of dopamine synthesis rather than an artefact of toxicity.

4.4 Discussion

The inhibition of honey bee worker reproduction by QMP enables queens to retain reproductive dominance over her workers (Hoover *et al*., 2003), a feature which is central to advanced eusociality (Wilson, 1971). In honey bees, dopamine is thought to be a key signalling molecule mediating QMP's constraint of reproduction in workers (Chapter three; Sasaki & Harano, 2010; Sasaki *et al*., 2021). The inhibition of ovarian development in QR workers has been widely assumed to be downstream of depression of brain dopamine by QMP, though prior to this study just one investigation formed the basis of this assumed causal relationship in honey bees (Dombroski *et al*. 2003).

In contrast with Dombroski *et al*., (2003), dopamine supplementation at 0.01 mg dopamine/ g food was not found to increase ovary activity levels in QL workers. The ineffectiveness of dopamine supplementation as a promotor of ovarian development in QL workers was consistent across the range of heightened dosage regimes. This discrepancy may be explained by the strains of bees used: the present study used European *A. mellifera,* while Dombroski *et al*., (2003) used Africanized *A. mellifera,* a strain known to activate their ovaries more readily (Zillikens *et al*., 1998). Alternatively, differences in the diet supplied to bees may explain this inconsistency. In this study, bees received a diet higher in protein (see Chapter two, section 2.1.1) than used by Dombroski *et al.* (2003), which may have facilitated ovary activation to such a degree that which dopamine supplementation was unable to elevate any further.

However, aspects of the experimental design may also contribute towards the results reported by Dombroski *et al*., (2003). Notably, the scoring of ovary activation level was not carried out blinded to dopamine treatment, meaning unconscious bias was not eliminated from the process.

As well as dopamine, administration of its precursor L-dopa was similarly not found promote ovarian development in QL workers at any dose tested. However, L-dopa supplementation also did not increase brain levels of dopamine. Decarboxylation of L-dopa to dopamine is accomplished by the enzymatic action of dopa decarboxylase (Figure 4.1; Neckameyer, 1996). It is possible that this enzyme is the rate limiting step in the biosynthesis of dopamine from L-dopa, with expression levels in the brain being the factor limiting the production of dopamine as opposed to L-dopa availability. Alternatively, oral intake of L-dopa may not have been directed to the brain, instead elevating dopamine titres in other tissues. To my knowledge, there are no comparable studies assessing the trajectory of dopamine biosynthesis from orally administered L-dopa in invertebrates. However, dopamine supplementation is commonly achieved through treatment with L-dopa as a therapy for Parkinson's Disease in humans (Nagatsua & Sawadab, 2009). Whilst L-dopa is better able to cross the blood brain barrier than dopamine (Haddad *et al*., 2018), the inclusion of additional pharmacological components is still required for effective transportation into the brain (Nagatsua & Sawadab, 2009). Assessment of dopamine levels in whole bees following L-dopa supplementation is needed to confirm whether this method of administration increases dopamine across a range of tissues. Because of its susceptibility to oxidation, it may be that the effects of L-dopa are transient or simply non-existent (Pendleton *et al*., 1996). If L-dopa treatment does increase dopamine levels in whole bees, the next steps would be to elucidate the tissue-specific effects. Likely destinations of orally administered L-dopa would be the gut, the hemolymph, or, as reported by Neckameyer (1996) for *D. melanogaster*, the central nervous system (CNS).

Assessment of internal dopamine levels of bees following treatment with dopamine itself would also be a useful tool to validate if, and where, elevations of dopamine levels occur. In attempting to replicate their findings, dopamine was administered to workers in their food using the methodology reported by Dombroski *et al*., (2003). Effects on internal dopamine levels were not measured by Dombroski *et al*., (2003), thus this would be key future work to validate the efficacy of this administration method for not only

this study but for previously published work. Similarly to L-dopa, dopamine is also highly susceptible to oxidation (National Center for Biotechnology Information, 2022), hence any effects on internal dopamine levels are likely to be transient. A possible alternative method which may lead to more stable elevations in dopamine levels would be to inhibit the degradation of dopamine by knocking down the expression of dopamine-N-acetyltransferase (the enzyme responsible for dopamine breakdown) using RNA interference. This being said, provision of the paper wasp *P. chinesis* with 1 mg/mL dopamine-spiked sucrose solution over a 10-day period reportedly increased brain dopamine levels (Sasaki *et al*., 2009), therefore this method may be expected to have the same effect in honey bees. Nonetheless, an investigation into the efficacy of dietary dopamine supplementation as a way to increase dopamine levels in this context would validate the use of this methodology in future manipulative experiments.

Despite uncertainty over the efficacy of dopamine supplementation methods, support for an involvement of dopamine in mediating ovarian development in honey bee workers comes from the reductions in ovary activity seen following inhibition of dopamine biosynthesis with iodotyrosine. Dopamine synthesis from the amino acid tyrosine requires hydroxylation to L-Dopa, primarily via the enzyme tyrosine hydroxylase (Figure 4.1). As an inhibitor of tyrosine hydroxylase, oral iodotyrosine application has been demonstrated to depress dopamine levels in whole *D. melanogaster* (Neckameyer, 1996), though tissue-specific effects are unclear. At a dose of 0.1 mg/mL, iodotyrosine treatment caused a reduction in ovarian development in honey bees with no effect on mortality, thus it is assumed that the repression of ovary activity is in response to dopamine depression specifically as opposed to general toxicity.

Reduced reproductive capacity following experimental depression of dopamine has been reported in other species, suggesting a common necessity of dopamine for basic reproductive function. In *D. melanogaster,* inhibition of dopamine synthesis resulted in inhibited ovarian development (Neckameyer, 1996) and failure to produce viable progeny (Pendleton *et al*., 1996), effects which were both reversed by co-treatment with L-dopa (Neckameyer, 1996; Pendleton *et al*., 1996). Similarly, iodotyrosine treatment at

0.15 mg/mL reduced numbers of oocytes and deposited eggs produced by virgin fire ant females, and this was also rescued by co-treatment with 0.15 mg/mL L-dopa (Boulay *et al*., 2001). The specific function of dopamine in the regulation of reproduction in honey bees is unclear. In some insects, dopamine is thought to stimulate the production and release of the gonadotropin JH by the corpora allata (CA), as is the case in the cockroach *Blatella germanica* (Cassier *et al*., 1993), the larval tobacco hornworm *Manduca sexta* (Granger *et al*., 1996), and *Drosophila* species (Gruntenko *et al*., 2005, 2007). However, JH does not appear to function as a gonadotropin in honey bees, and it is also unclear whether it is under regulation by dopamine (Rodrigues & Flatt, 2016). Although dopamine appears to be required for reproduction in honey bees, its pathway of action is likely to differ to that seen in solitary insects, perhaps involving direct interaction with the ovary (Chapter three).

Regardless of the underlying mechanism, dopamine's apparent positive regulatory role in ovarian development in honey bees makes it a likely target for QMP in the regulation of worker reproduction. QMP is known to depress brain dopamine levels (Beggs *et al*., 2007; Harris & Woodring, 1995; Sasaki & Nagao, 2001), with HVA in particular being the component at least partially responsible for this depression (Beggs & Mercer, 2009). Here, it is demonstrated that treatment of QL workers with a dopamine synthesis inhibitor led to reductions in ovary activity comparable with those induced by QMP, directly implicating QMP's lowering of dopamine as having a direct negative effect on reproduction in workers.

If dopamine levels are a major factor governing ovarian development, this may appear to be at odds with the finding that dopamine supplementation did not overcome QMP's inhibitory effects. Possible limitations of dopamine supplementation methods that may play into this have been discussed above. However, it is perhaps unsurprising that QMP's repression cannot be overcome simply via the restoration of dopamine given the multiple levels of functional redundancy within QMP's components (Princen *et al*., 2019). QMP contains a blend of five major compounds: (2E)-9-oxo-dec-2-enoic acid (9-ODA), both enantiomers of (2E)-9-hydroxydec-2-enoic acid (9-HDA), methyl-4-

hydroxybenzoate (HOB) and the component responsible for lowering dopamine, homovanillyl alcohol (HVA) (Slessor *et al*., 1988). Exposure of honey bee workers to HVA alone results in partial inhibition of reproduction (Princen *et al*., 2019), while exposure to 9-ODA or 9-HDA causes equivalent levels of repression to that seen with the five-component blend (Princen *et al*., 2019). This indicates that the depression of brain dopamine by HVA is likely just one process among several redundant mechanisms acting to constrain worker reproduction in the presence of QMP. Two competing evolutionary theories may explain this existence of multiple redundant mechanisms – the queen signal hypothesis and the queen control hypothesis (refer Section 1.4, Introduction). Support for each theory in light of these findings is discussed further in Section 7.5, General Discussion.

In QL workers, the loss of QMP's depressive effects on dopamine is presumably a factor which enables ovarian development in some workers in the absence of a queen. Attempts to further elevate dopamine in QL workers in this study did not increase rates of ovary activation (in contrast with Dombroski *et al*. 2003), though this is perhaps unsurprising. While a minimum level of dopamine has been shown to be necessary for normal ovarian development (i.e. in *Drosophila;* Neckameyer, 1996; Pendleton *et al*., 1996), it is unlikely the limiting factor in the absence of QMP's depressive effects. For instance, in a QL hive, dopamine levels in workers are no longer being suppressed by QMP, yet still only 24-30% of workers respond to the queen's absence by activating their ovaries (Jay, 1968; Miller & Ratnieks, 2001). This is partly because the propensity of individual workers to activate their ovaries in a QL setting is under the influence of additional factors, including ovariole number (Ronai *et al*., 2017) and genetic relatedness to the queen (Rojek & Kuszewska, 2022). This is illustrative of the fact that multiple complex mechanisms interact to govern ovarian development in adult worker honey bees. While I have found evidence to support the idea that QMP's modulation of dopamine plays a part in maintaining the sterility of honey bee workers, it also highlights that this single signalling pathway is just one among several different reproductive constraints (refer Section 1.2, Introduction).

In summary, this chapter demonstrates that dopamine is likely required for ovarian development in adult worker honey bees, supporting the hypothesis that QMP's control over dopamine titres (demonstrated in Chapter three and in previously published studies) is a pathway involved in the queen's repression of worker reproduction. Due to the uncertainty over the final destination of dopamine supplementation treatments (i.e. whether dopamine was elevated in the brain, the hemolymph, or not at all following treatment), this chapter cannot shed light on whether dopamine's role in reproduction is driven by levels in the brain, in the hemolymph, or a combination of both (Chapter three). As such, the findings from this chapter could be interpreted with more nuance if the effects of dopamine supplementation and inhibition treatments on biogenic amine titres in specific tissues were known. Investigation into the tissue-specific effects of the dopamine supplementation and inhibition methods used in this study on biogenic amine titres is therefore also a key area for future research.

In Chapter three, a role of octopamine in mediating reproductive constraint was also speculated, and future work addressing the causal relationship between octopamine titres and ovary development, as has been carried out here for dopamine, would also be a key avenue for future research.

Chapter 5

Dose-dependent effects of QMP on reproduction

5.1 Introduction

The monopolization of reproduction by one or several reproductive females in a colony is a defining feature of eusociality (Wilson, 1975. The reproductive skew in honey bee societies is particularly extreme, with colonies of tens of thousands of facultatively sterile female workers headed by a single highly fecund queen responsible for laying up to 1,500 eggs per day (Snodgrass, 1956). Irreversible differences in queen-worker physiology are established during larval development (Cameron *et al*., 2013; Kucharski *et al*., 2008), which facilitate this high reproductive output from queens. In a process triggered by the feeding of royal jelly (Dixon & Shuel, 1963), queens develop considerably larger ovaries than workers, consisting of 120-200 ovarioles and possessing a fully developed spermatheca (Figure 1.1; Snodgrass, 1956; Linksvayer *et al*., 2011). Worker ovaries, on the other hand, lack a functional spermatheca and consist of just 2-12 ovarioles (Figure 1.1; Snodgrass 1956), a result of programmed cell death in early development (Capella & Hartfelder, 1998; Hartfelder & Steinbrück, 1997). These developmental constraints severely reduce the reproductive capacity of workers relative to queens, though workers still retain some ability to reproduce.

In addition to developmental constraints on worker reproduction, queens impose further restrictions on worker reproduction during adulthood via QMP (Hoover *et al*., 2003). There is considerable debate as to whether QMP, and queen pheromones more generally, chemically control worker reproduction or simply signal the presence of a fecund queen, two hypotheses known as the 'queen control' and 'queen signal' hypothesis (refer Section 1.4, General Introduction; Keller & Nonacs, 1993; Kocher & Grozinger, 2011; le Conte & Hefetz, 2008; Strauss *et al*., 2008). Under the honest signal hypothesis, it is thought that workers under the presence of a fecund queen benefit more from rearing the queen's brood (i.e. their siblings) than attempting to reproduce

themselves (Keller & Nonacs, 1993). As such, queen pheromones should be selected for their ability to reliably or 'honestly' signal the queen's fecundity (Oi *et al*., 2015). On the other hand, the queen control hypothesis predicts that QMP has evolved to target pathways mediating reproduction to chemically enforce sterility in workers (Reviewed by Kocher & Grozinger, 2011).

In line with the queen signal hypothesis, the production of QMP is tightly linked with the queen's reproductive state, depicting QMP as an honest signal of fecundity (Kocher *et al*., 2009). For instance, the quantity and chemical composition of QMP produced differs between virgin and mated queens (Plettner *et al*., 1997). QMP production also varies between laying vs non-laying mated queens (Kocher *et al*., 2008), with the number of drones she has mated with (Richard *et al*., 2007), and with semen insemination volume (Niño *et al*., 2012).

An alternative though not necessarily mutually exclusive hypothesis, the queen control hypothesis, predicts that queen pheromones have evolved to target pathways mediating reproduction to exert direct control over workers (Reviewed by Kocher & Grozinger, 2011). QMP is highly derived from the ancestral class of cuticular hydrocarbons that function as queen pheromones in other hymenopterans (refer Section 1.3, General Introduction). Unlike these ancestral pheromones, QMP inhibits the reproduction not only of honey bees, but of a broad range of unrelated species (Lovegrove *et al*., 2019). For instance, QMP has been found to reduce reproductive capacity in virgin *D. melanogaster* females (Camiletti *et al*., 2013), a species of ant (Carlisle & Butler, 1956), a house fly (Nayar, 1963), a termite (Hrdy *et al*., 1960), and a prawn (Carlisle & Butler, 1956). These broad phylogenetic effects suggest that QMP may induce sterility by targeting pre-existing ancestral pathways involved in regulating reproduction, depicting this pheromone as an agent of reproductive control. Though much is still unknown about the process by which QMP constrains worker reproduction, the pheromone's modulation of dopamine (and possibly octopamine; Chapter three) appears to be a factor inhibiting ovary development in workers (Chapter four). In fact, the direct modulation of brain dopamine by the QMP component HVA is sometimes cited in

support of the reproductive control hypothesis (Beggs *et al*., 2007; Beggs & Mercer, 2009; Kocher & Grozinger, 2011), as it points to QMP being able to manipulate an important signalling molecule to induce sterility. If QMP directly manipulates fundamental reproductive pathways, an unexplored question is how the queen is able to avoid self-repression with her own pheromone. Reconciling this apparent contradiction is thus a useful addition to the debate surrounding the queen control vs queen signal hypotheses.

The mean quantity of QMP found in the mandibular glands of a laying queen is 220 µg, consisting of 150 µg 9-ODA, 55 µg 9-HDA, 13 µg HOB, and 1.5 µg HVA, or one queen equivalent (QE) (Slessor *et al*., 1988). One QE is sufficient to induce the full retinue response of workers (Slessor *et al*., 1988), inhibit the production of queen cells (Winston *et al*., 1990), and repress the ovary activity of tens of thousands of workers in a hive (Hoover *et al*., 2003). Although the majority of the QMP produced by the queen is disseminated across the colony by workers (Naumann *et al*., 1991), 36% of these daily secretions are re-internalised by the queen, most likely via ingestion (Figure 1; Naumann *et al*., 1991). Despite this, the queen still manages to retain extraordinarily high levels of fecundity, being seemingly immune to the effects of her own pheromone.

One possible explanation for QMP's lack of inhibitory effects in queens may lie in the differing degrees to which queens and workers are exposed. Once disseminated throughout the tens of thousands of workers in the hive, the dose of QMP received by individual workers is presumably very small compared with the approximately 0.36 QE internalised by queens (36% of 1 QE; Naumann *et al*., 1991). Hormesis, a commonly observed dose-response phenomenon, is a term predominantly applied to the field of medicine describing biphasic dose-response relationships of U-shaped or inverted U-shaped nature (Calabrese & Baldwin, 2002). The repressive effects of QMP may adhere to this, whereby in low doses it inhibits ovary development, but is ineffective at high doses due to overstimulation or over compensatory mechanisms. Here, I test the hypothesis that QMP's inhibition of reproduction is biphasic with respect to dose by

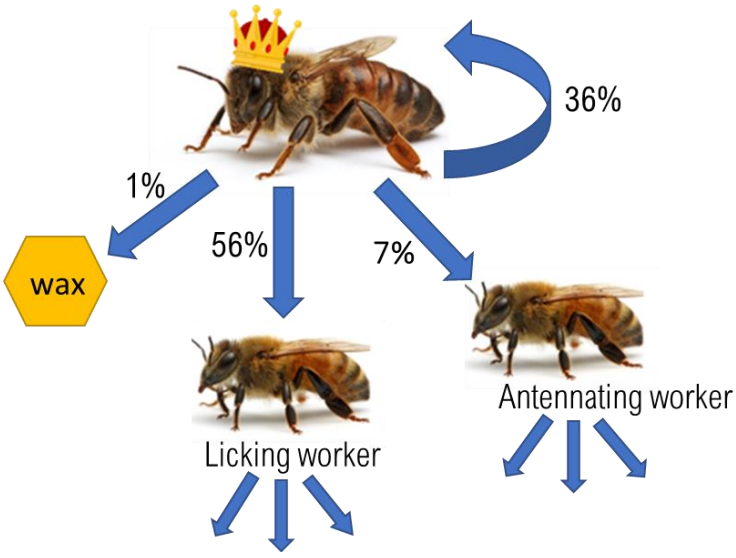

Figure 5.1 Schematic of modelled QMP transmission throughout the hive. Adapted from Naumann *et al*., (1991).

exposing workers to QMP at a range of concentrations and observing the impact on their ovary development.

To gain further insight into possible mechanisms involved in this U-shaped dose response, I also examine the effect of different QMP doses on some of our best-understood processes mediating reproductive constraint (refer Section 1.5, General Introduction). Based on current understanding, active Notch signalling in the ovary is the proximate inhibitory mechanism in response to low-dose QMP exposure (Section 1.5.1; Duncan *et al*., 2016), and the depression of dopamine and octopamine is likely involved in the upstream sequence of events (Chapter three, Chapter four). Interestingly, despite continual QMP exposure, brain dopamine levels are elevated in queens compared with queenright (QR) workers (Sasaki *et al*., 2012), and Notch signalling is inactive in the ovary (Duncan *et al*., 2016). This indicates that at elevated doses, QMP's effects on biogenic amines and Notch activity may be lost, which may underpin the lack of reproductive inhibition. To test the hypothesis that QMP's effects on Notch activity and biogenic amine modulation are dose-dependent, I also measure Notch activity in the ovaries of workers exposed to different doses of QMP, as well as brain levels of dopamine and octopamine. To my knowledge, this study is the first of its kind to ask the question of

how honey bee queens evade self-repression by QMP, and aims to empirically test one potential mechanism explaining the caste-specific responses of queens and workers to this pheromone.

5.2 Methods

5.2.1 Microcolony set-up and experiment overview

A. mellifera hives were maintained as stated (Section 2.1.1) and laboratory microcolonies were setup and maintained in cages as described previously (Section 2.1.1). Experimental cages each contained 80 workers and were exposed to QMP at varying doses. After 10 days the ovaries of all remaining workers in each cage were dissected, imaged and scored to assess ovary activity levels as described previously (Section 2.2). Ovaries and brains from a subset of five randomly selected bees per cage were collected, snap frozen in liquid nitrogen and stored at -80°C for later molecular analysis. Four experimental replicates were carried out for each cage between July-August 2020.

5.2.2 QMP treatments

Synthetic QMP was supplied as described previously (Section 2.1.2) but at a wider range of concentrations. As in other chapters (Chapter three, Chapter four, Chapter six) and previous work (Duncan *et al*., 2016), QMP was supplied to some treatment cages at 0.01 QE/µL, as this concentration has been shown to inhibit the reproduction of 80-100 workers in a microcolony. Other cages received QMP at the elevated doses of 0.1 and 1 QE/µL, and QL microcolonies received just solvent control (0 QE/µL). Microcolonies received QMP treatments as 10 µL droplets which were replaced daily (as described in Section 2.1.2). Therefore, the total QE received by microcolonies each day was 0, 0.1, 1 or 10.

5.2.3 HPLC-FLD

To test the hypothesis that QMP's modulation of biogenic amines is dose-dependent, dopamine and octopamine were quantified in brain samples using High Performance

Liquid Chromatography with Fluorescent Detection (HPLC-FLD) as described previously (Section 2.3).

5.2.4 Quantitative RT-PCR

To determine whether QMP's regulation of oogenesis via Notch signalling is dose-specific, expression of the genes *bHLH2* and *Her* were measured in the ovary as a proxy for Notch activity using RT-qPCR. These two E(spl)-C genes (enhancer of split complex; Duncan & Dearden, 2010; Jennings *et al*., 1994) are targets of Notch signalling, and have previously been implicated in Notch-mediated ovarian development (Duncan *et al*., 2016).

RNA was extracted from individual snap-frozen pairs of ovaries using RNAqueous micro kits (ThermoFischer) with removal of gDNA with DNAse treatment. 100 ng RNA or the maximum amount obtained was reverse transcribed and a 1 in 5 dilution of this cDNA was used as a template for RT-qPCR. Differences in cDNA quantities between samples are standardised by comparing the relative expression of genes of interest to that of the reference or 'housekeeper' gene (described in full below). Oligonucleotide primers used were also designed as described in (Duncan *et al*., 2016). Oligonucleotide primer sequences are provided in Appendix H, Table H.1**.**

RT-qPCR was carried out using a BioRad CFX RT-PCR detection system. Each reaction contained 15 µL SsoFast EvaGreen PCR mastermix**,** 5 ng of cDNA, and 300 nM of each primer, and each measurement was carried out in duplicate. Gene expression for every condition (every ovary score within each QMP dose treatment) was measured for three biological replicates. However, it was not possible to obtain three biological replicates of every single combination of ovary score and QMP dose due to the frequency distribution of ovary scores within each QMP treatment (i.e. Figure 5.4). For instance, no workers sampled for RT-qPCR receiving 0.01 QE/ µL QMP had an ovary score of 3 due to the inhibition of ovary activity. A breakdown of the number of biological replicates for each condition is provided in Appendix H, Table H.2.

Expression of target genes *Her* and *bHLH2* was normalised to the relative quantities of the reference gene *Rpn2* using the Pfaffl method (Pfaffl, 2001). The sample with the highest Ct value was used as a calibrator sample to calculate ΔCt. Initial reference gene selection based on their stability of expression among ovary samples was carried out as in (Duncan *et al*., 2016). Insufficient RNA was obtained from individual ovaries to normalise expression of genes of interest against more than one housekeeping gene. The use of *Rpn2* only as a housekeeping gene was validated by an analysis of previous gene expression data normalised to either *Rpn2* alone or the geometric mean of *Rpn2* with a second housekeeper, *mRPL44* (Appendix H, Figure H.1). Gene expression data was $\log_{10}$ transformed prior to analysis.

5.2.5 Data analysis

All statistical analyses were carried out using R. Details of packages used for analysis are found in section 2.4. For all analyses, QMP dose was considered an ordinal variable, as the number of doses tested was insufficient to be considered a continual variable.

Food intake and survival rates were compared across treatment conditions to establish whether there were any compounding effects of QMP doses. Mean food intake per bee per day was compared across treatment conditions by fitting LMMs with QMP dose as the fixed effect. To account for repeated measures, day was included as a random effect and nested within replicate. Statistical significance of QMP dose on food intake was determined by comparing the likelihood ratio of the full model to the null model containing no fixed effect. Survival distributions were compared between treatment conditions using CPH models with mixed effects, where QMP dose was included as a fixed effect and replicate as a random effect. The assumption of proportional hazards was verified through visual examination of correlated scaled Schoenfield residuals with time to test for independence. CPH models were used to predict Hazard Ratios (HR) and confidence intervals (CI) for fixed effects. HRs are presented as HR (±95% CI).

To compare ovary activation rates across different QMP doses, CLMMs were fitted with ovary score as the ordinal response variable, QMP dose as the fixed effect and replicate as the random effect. Statistical significance was obtained by comparing the likelihood ratio of the full model to the null model containing no fixed effect. *Post-hoc* testing was carried out by computing least-squares means to determine the significance of all pairwise comparisons, and P-values were Tukey-adjusted.

Differences in gene expression between ovaries of each score within each QMP dose treatment were analysed using LMMs with ovary score, QMP dose and their interaction as fixed effects and replicate as a random effect. Statistical significance of each fixed effect was computed by comparing likelihood ratios between the maximal model and the model without the fixed effect of interest. *Post-hoc* testing was carried out by computing estimated marginal means on the best-fitting model as indicated by AIC score.

Brain levels of dopamine and octopamine were compared between QMP dose treatments by fitting GLMMs with a gamma distribution and an inverse link function for each biogenic amine. QMP dose was included as the fixed effect and replicate and sample preparation block were included as random effects. The statistical significance of QMP dose on each biogenic amine was obtained by comparing the likelihood ratio of the full model to that of the null model containing no fixed effects.

5.3 Results

5.3.1 QMP treatments do not affect food intake or survival

Food intake was not affected by QMP dose (Figure 5.2; LMMs; $\chi 2 = 1.33$, df = 3,36, p = 0.72), confirming that there were no nutritional differences in workers between treatment conditions that could explain differences in ovary activity. Across all four replicates, survival rates of caged workers were very high, with cage survival ranging between 93-100% (Figure 5.2). Exposure to QMP at 0.01 QE/µL and 1 QE/µL was associated with respective increased mortality risks of 12% and 30% compared with QL workers (CPH; 0.01 QE/µL: HR = 1.12 ± 0.52, z = 2.17, p <0.05; 1 QE/µL: HR = 1.30 ± 0.51, z = 2.58, p <0.05), though due to the overall high survival in each treatment condition this is not deemed disruptive to experimental results. Full *post-hoc* pairwise comparisons computed using estimated marginal means can be found in Table 5.1.

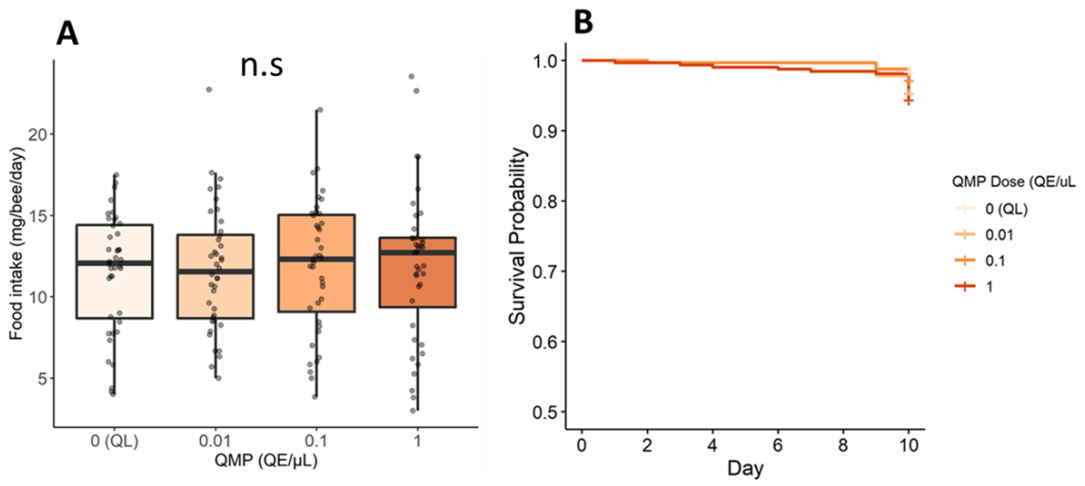

Figure 5.2 Effects of QMP doses on food intake and survival. A. Mean food intake per bee per day at different QMP dose treatments. Food intake was recorded daily for each treatment cage (n = 10 days), for which there were four replicates (n = 40 for each treatment). Food intake was consistent across different levels of QMP exposure as denoted by 'n.s' (LMMs). **B.** Survival probability distributions of cages of 80 bees over 10 days of exposure to different concentrations of QMP. Data shown represents the pooled survival distributions over four experimental replicates. See table 5.1 for *post-hoc* comparisons of survival between QMP treatments

Contrast (QE/µL)	Hazard Ratio	SE	df	Z-ratio	P-value
0-0.01	-1.12	0.52	Inf	-2.17	0.13
0-0.1	-0.59	0.56	Inf	-1.06	0.71
0-1	-1.30	0.51	Inf	-2.58	0.05*
0.01-0.1	0.53	0.42	Inf	1.25	0.59
0.01-1	-0.19	0.35	Inf	-0.53	0.95
0.1-1	-0.71	0.41	Inf	-1.75	0.30

Table 5.1 list of *post-hoc* pairwise comparisons for survival rates between different QMP exposure treatments.

5.3.2 Brain dopamine and octopamine are not affected by QMP dose

It was hypothesised that QMP's depression of biogenic amines may be lost at high doses, leading to misinterpretation of QMP's signal and impeding reproductive constraint. No evidence was found in support of this hypothesis, as brain levels of dopamine and octopamine in 10-day old workers were not affected by QMP at any dose (Figure 5.3; GLMMs; Dopamine: $\chi^2 = 2.79$, df = 3,74, p=0.42; Octopamine: $\chi^2 = 2.83$, df = 3,74, p=0.42). However, this includes a lack of expected depression by QMP at the low dose (0.01 QE/µL), as has been observed previously for octopamine in the laboratory (Chapter three) and for both dopamine and octopamine in QR hives (Chapter three).

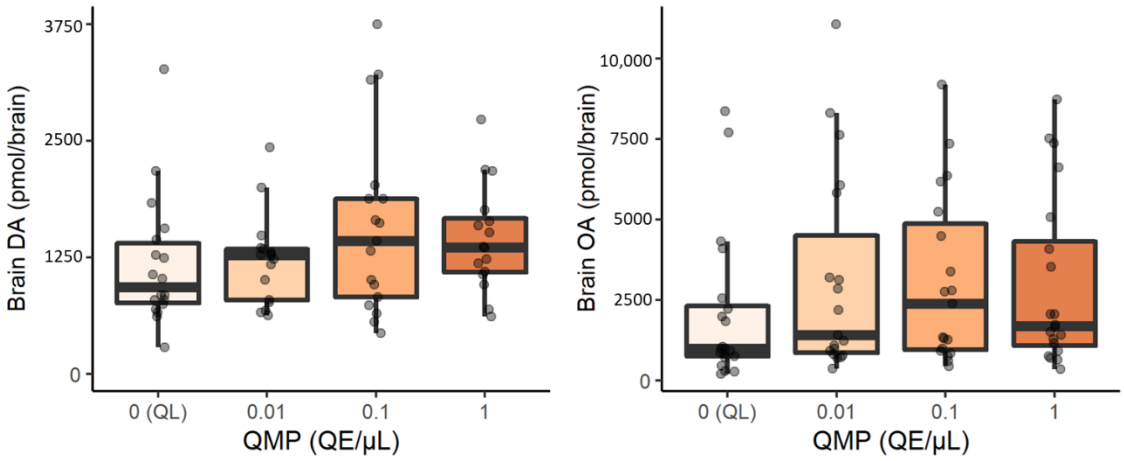

Figure 5.3 Brain biogenic amine levels at different QMP doses. HPLC-FLD quantification of biogenic amines in the brains of 10-day old worker bees following exposure to QMP at a range of concentrations. Brains were sampled from five randomly selected bees per treatment cage over four replicates (n=20 total for each QMP dose). Pairwise GLMM model comparisons showed that levels of **A.** dopamine and **B.** octopamine were not significantly affected by QMP dose, as denoted by "n.s".

5.3.3 QMP's inhibition of worker reproduction is lost at high doses

Ovary development was affected by QMP in a dose-dependent manner (Figure 5.4). CLMM model comparisons demonstrated that QMP dose significantly affected ovary activity levels in workers (CLMM; $\chi^2 = 62.16$, df = 3,1034, $p < 0.001$). Consistent with other work (Chapter three, Chapter four, Chapter six, Duncan *et al*., 2016), worker reproduction was repressed by synthetic QMP at a dose of 0.01 QE/ µL. At this lower dose, the percentage of workers possessing fully mature oocytes (ovary score of 3; Figure 2.2) was reduced from 30% in QL workers to 9%. *Post-hoc* testing using least marginal means on the full model showed this decrease in ovary activity levels between 0 QE/µL and 0.01 QE/µL was statistically significant (Z = -6.808, $p < 0.001$). All *post-hoc* pairwise comparisons can be found in Table 5.2.

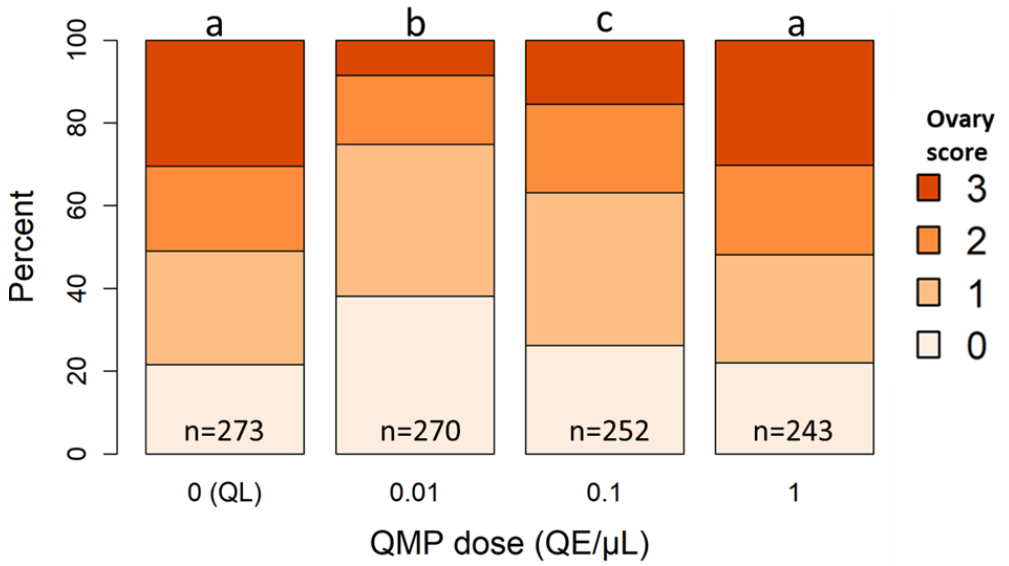

Figure 5.4 Dose-specific effects of QMP on ovary activity. Ovary activity levels of 10-day old workers maintained in the absence of QMP (0 QE/µL – QL) or in the presence of QMP at different concentrations. At 0.01 QE/µL, QMP represses ovary activity relative to QL workers, but this repression subsides with increasing QMP dose. Ovary activity is presented as the percentage of workers within each treatment condition with each ovary score (ranging from 0-3), where a higher score (darker shade) relates to a higher degree of development. N values for each treatment represent totals from four pooled replicates and are displayed at the base of each bar. Statistically significant pairwise comparisons ($p < 0.05$) are denoted by bars not sharing a letter, where *post-hoc* testing was carried out on the full CLMM using least squares means. For full results from *post-hoc* testing see Table 5.2.

Contrast (QE/µL)	Estimate	SE	df	Z-ratio	P-value
0-0.01	-1.08	0.16	Inf	-6.81	<0.0001***
0-0.1	-0.55	0.16	Inf	-3.47	0.003**
0-1	-0.02	0.16	Inf	-0.14	0.999
0.01-0.1	0.53	0.16	Inf	3.37	0.004**
0.01-1	1.06	0.16	Inf	6.47	<0.0001***
0.1-1	0.53	0.16	Inf	3.22	0.007**

Table 5.2 list of *post-hoc* pairwise comparisons for ovary score distributions between different QMP exposure treatments.

Interestingly, as QMP dose was increased above 0.01 QE/µL, its repression of worker ovary activity subsided. At a dose of 1 QE/µL QMP's inhibitory effects were completely absent, and *post-hoc* testing showed that ovary activity levels did not significantly differ at this high dose from that seen at 0 QE/µL (Z = -0.14, p = 0.999). The intermediate QMP dose of 0.1 QE/µL represented an intermediate level of repression lying between full repression at 0.01 QE/µL and the absence of repression at 1 QE/µL (see Figure 5.4 and Table 5.2).

5.3.4 Notch signalling underlies reproductive constraint irrespective of QMP dose

In keeping with previous work (Duncan *et al*., 2016), Notch signalling appears to be the process actively constraining ovarian development (Figure 5.5). While this has been demonstrated with pooled samples before, this is the first time this has been shown for individual bees. Expression of *Her* and *bHLH2* in the ovary, proxies for Notch activity, negatively correlated with the degree of ovarian development irrespective of QMP dose received (Figure 5.5). Model comparisons demonstrated QMP dose to have no significant effect on expression of either *Her* (LMMs; $\chi^2 = 8.10$, df = 9,28, p = 0.52) or *bHLH2* (LMMs; $\chi^2 = 7.40$, df = 9, 28, p = 0.60). Expression of both genes was instead more closely linked with ovary score, and this was statistically significant (LMMs; *Her:* $\chi^2 = 17.79$, df = 9, 28, p <0.05; *bHLH2:* $\chi^2 = 28.23$, df = 9,28, p <0.001). The best-fitting model for the data (as determined using AIC values) included just ovary score as the sole fixed effect, hence this is also presented graphically with pooled QMP doses (Figure 5.5). Pairwise *post-hoc* comparisons using estimated marginal means were carried out on this

reduced model and are presented in both Figure 5.5 and Table 5.3. There was no evidence for an interaction effect between QMP dose and ovary score on *Her* or *bHLH2* expression (LMMs; *Her:* $\chi^2 = 6.71$, df = 6,31, p=0.35; *bHLH2:* $\chi^2 = 6.39$, df = 6,31, p=0.38), indicating that Notch activity consistently regulates development in the ovarian tissue regardless of upstream changes to environmental cues or signalling pathways that may arise from different QMP doses.

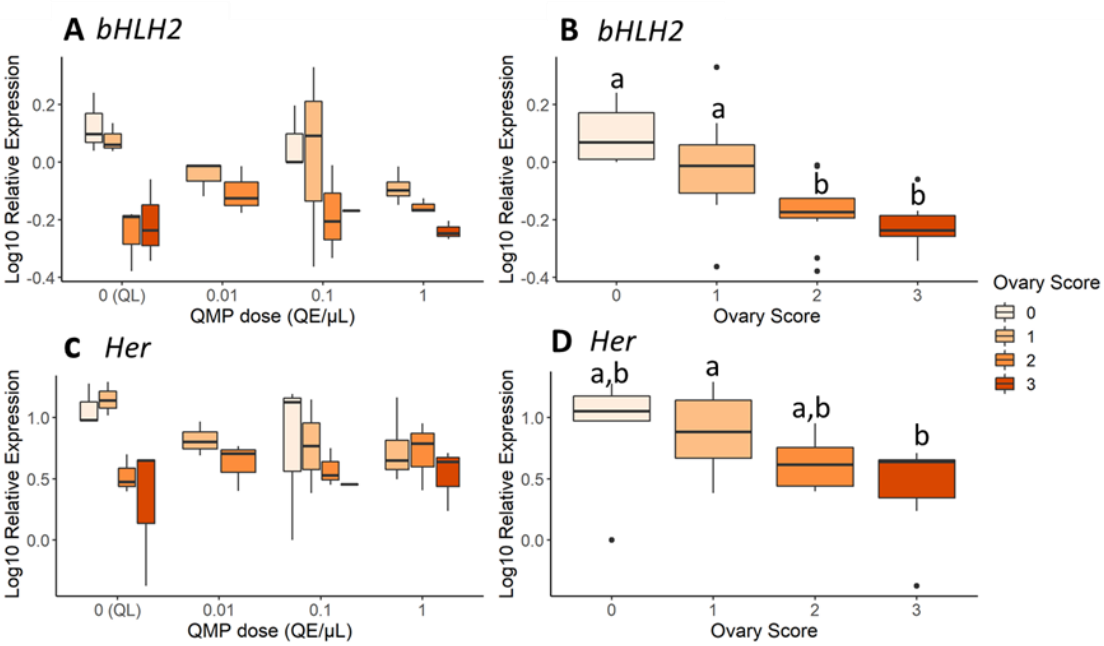

Figure 5.5 *Her* and *bHLH2* ovary expression by ovary score and QMP dose. Expression of two genes from the E(spl)-C complex in worker ovaries measured using RT-qPCR as a proxy for Notch activity. Gene expression of **A.** *bHLH2* and **C.** *Her* was measured for each ovary score within each QMP dose treatment condition in triplicate where possible (see supplementary Table H.2, Appendix H for sample sizes). LMMs revealed ovary score but not QMP dose to significantly affect expression of both genes, hence expression of **B.** *bHLH2* and **D.** *Her* is also presented for each ovary with QMP dose treatment conditions pooled. Relative expression of both *bHLH2* and *Her* decrease with increasing ovary scores. *Post-hoc* tests were carried out using estimated marginal means on the best fitting model (lowest AIC) with ovary score as the sole fixed effect. Statistically significant pairwise comparisons ($p<0.05$) are denoted on plots **B.** and **D.** by boxplots not sharing a letter. *Post-hoc* pairwise comparisons are also reported in Table 5.3

bHLH2					
Contrast	**Estimate**	**SE**	**df**	**T-ratio**	**P-value**
0-1	0.11	0.07	33.9	1.69	0.34
0-2	0.27	0.07	33.8	3.93	0.002**
0-3	0.31	0.08	33.7	4.14	0.001**
1-2	0.16	0.05	34.0	2.90	0.03*
1-3	0.20	0.06	32.9	3.29	0.01*
2-3	0.04	0.06	32.7	0.73	0.89
Her					
Contrast	**Estimate**	**SE**	**df**	**T-ratio**	**P-value**
0-1	0.03	0.16	32.3	0.21	0.99
0-2	0.25	0.16	32.2	1.57	0.41
0-3	0.45	0.18	32.6	2.52	0.08
1-2	0.22	0.13	31.4	1.71	0.34
1-3	0.42	0.15	30.7	2.89	0.03*
2-3	0.20	0.15	31.0	1.40	0.51

Table 5.3 List of *post-hoc* pairwise comparisons for the expression of *bHLH2* and *Her* between different ovary scores

5.4 Discussion

The absence of self-sterilizing effects of QMP in queens is a phenomenon which has received little research attention, yet is central to the validity of the queen control hypothesis; If QMP is a direct suppressor of reproduction, what mechanisms protect queen fecundity? Here, I provide empirical evidence that QMP's inhibition of reproduction is lost at high doses, consistent with the hypothesis that differential exposure levels between queens and workers is a mechanism underlying caste-specific repressive effects of QMP. This provides our first insight into one proposed mechanism by which queens evade self-sterilization, though alternative mechanisms will also be discussed which remain to be tested.

The inhibition of reproduction by QMP at low doses but loss of function at high doses resembles a hormetic response (Figure 5.6). Characterized by biphasic dose response relationships, hormesis is generally driven by overstimulation or over compensatory mechanisms (Calabrese & Baldwin, 2002). The processes underlying QMP's mediation of reproduction are complex and involve multiple stages (refer General Introduction, Section 1.5), and such a dose-response relationship could occur at any point along this

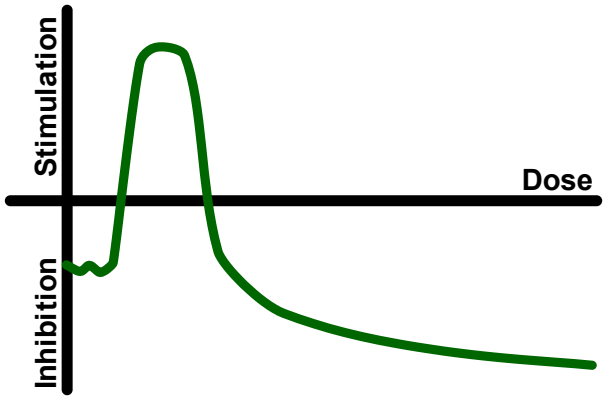

Figure 5.6 Hormesis dose-response curve. Hormesis is characterised by bi-phasic, U-shaped or inverse U-shaped dose responses. QMP's repression of ovary activity resembles a hormesis dose-response curve, with no inhibition of ovary activity at a dose of 0, inhibition at low doses, and loss of inhibition at high doses (Figure 5.4).

signalling pathway to drive the loss of reproductive inhibition at high doses. In the ovary itself, Notch signalling is the mechanism that actively blocks development in workers exposed to QMP at normal levels (Duncan *et al*., 2016). In the absence of QMP, Notch activity is negatively correlated with worker ovary development, but is lowest in queens in spite of their re-internalisation of QMP (Duncan *et al*., 2016). It is tempting to conclude that dysfunctional Notch activity at high QMP doses facilitates queen ovary activity. However, the finding that Notch signalling was the mechanism controlling ovary development even in workers exposed to high doses of QMP suggests that the hormetic processes driving the loss of inhibitory effects of QMP at high doses occur upstream.

As previously discussed, the depression of dopamine and possibly octopamine is involved in mediating QMP's repression of ovary development (Chapter three, Chapter four). Despite the re-internalisation of QMP, queens have elevated brain dopamine levels relative to workers (Sasaki *et al*., 2012). It was thus hypothesised that QMP's depression of biogenic amines may be lost at high doses, facilitating ovary development despite the presence of QMP. The QMP component HVA is thought to modulate dopamine levels by directly interacting with AmDop3 receptors (Beggs & Mercer, 2009), hence overstimulation of these receptors by HVA at high QMP doses could lead to disinhibition of dopamine. Brain levels of both dopamine and octopamine were unaffected by QMP at any dose, providing no evidence for an involvement of these

biogenic amines in QMP's loss of function at high doses. However, the lack of depressive effects of QMP observed at low doses despite its inhibition of reproduction reinforces previously discussed experimental limitations (Chapter three). In particular, sampling workers on day 10 is likely too late to observe differences in biogenic amines that precede changes in ovary development; Previous work reporting reductions in brain dopamine by QMP sampled laboratory-maintained workers at two-days old (Beggs *et al*., 2007). A hormetic dose-response of key biogenic amines to QMP therefore cannot be ruled out as the mechanism underlying dose-specific differences in reproductive repression, and future studies sampling workers on day two of different QMP exposure regimes may be a more informative means of testing this.

Although the underlying mechanism is unclear, QMP's demonstrated dose-specific effects on reproduction supports the hypothesis that a hormetic dose-response relationship is a driver of caste-specific differences in QMP's repressive effects. However, this represents just one piece of evidence in favour of this hypothesis, and further study is required to test this more definitively. Firstly, the hypothesis rests heavily on the assumption that queens are exposed to QMP in greater quantities than workers, though empirical support for this is limited. One study in which 36% of QMP secretions were found to be re-internalized by the queen, likely via ingestion, forms the basis of this assumption (Naumann *et al*., 1991), with no other studies carried out on this subject to my knowledge. However, the authors point out that the estimated queen re-internalisation rate is the weakest element in their model of QMP transmission among the hive due to technical limitations of the model (Naumann *et al*., 1991). Obtaining an accurate measure of A. QMP re-internalisation rates of queens and B. QMP exposure levels of workers would provide a more robust foundation for the assumed heightened exposure of QMP in queens relative to workers. This would be difficult to study, but could theoretically be achieved using radio-labelled diet components fed to the queen which would be incorporated into QMP during synthesis by the queen.

Another key factor to consider is whether there are differences in the mode of QMP exposure between queens and workers that could explain QMP's caste-specific effects.

In workers, QMP is picked up by nurses attending the queen (Allen, 1955) and distributed through the colony via worker-worker interactions such as antennation, grooming and trophallaxis (Naumann *et al*., 1991). Evidence points to gustatory exposure being a predominant route of QMP detection necessary for the inhibition of ovary activity in honey bees, as preventing direct physical contact with QMP (Lovegrove *et al*., 2020) or restricting worker-worker trophallaxis in the hive (Katzav-Gozansky *et al*., 2004) lifts the repression of reproduction. The detection of labelled 9-ODA in the hemolymph and gut of queens suggests that ingestion is the most probable route of QMP re-internalisation (Naumann *et al*., 1991), thus gustatory exposure is also the likely mode of QMP detection in queens. However, this has not been empirically tested, and if alternative exposure routes such as absorption through the cuticle are instead the main route of re-internalisation, these discrepancies in QMP uptake between queens and workers could in themselves represent a mechanism of self-sterilization evasion in queens.

The queen signal hypothesis posits that QMP acts as an honest indicator of queen quality (Keller & Nonacs, 1993; Kocher & Grozinger, 2011), and this is supported by empirical evidence demonstrating equivalence between queen QMP production and aspects of reproductive potential (Kocher *et al*., 2008, 2009; Niño *et al*., 2012; Plettner *et al*., 1997; Richard *et al*., 2007). That QMP is not repressive at high doses presents an interesting possibility on the causality of QMP production and fecundity; Perhaps increased QMP production actually precedes queen ovarian maturity post-mating, and the heightened QMP exposure of queens is a necessary facilitator of oocyte development. Put simply, could QMP production be a cause rather than a consequence of high fecundity in queens? Such a hypothesis is highly speculative, and rests on previously discussed assumptions that remain to be validated such as whether QMP exposure truly is heightened in queens and whether the mode of uptake is comparable to workers. Nonetheless, this is a novel angle from which to consider the evolution of QMP signalling which joins together the queen signal and queen control hypotheses in an intriguing way. Through its dose-specific effects, QMP could hypothetically serve as both a positive

and negative regulator of reproductive control, rendering it also an innately honest signal of fecundity.

A means of testing the causality of QMP exposure and ovary development in queens would be to look to queens themselves. An ideal experiment would use RNA interference (RNAi) to knock down QMP production in queens, allowing external QMP exposure to be manipulated at a range of doses and the effects on queen ovary development examined. However, as the genes involved in the biosynthesis of QMP have not been fully elucidated, the specific genes that would need to be targeted for knock down are unclear. Surgical removal of the mandibular glands, the site of QMP synthesis, would be an alternative approach to restrict queen QMP production. Mandibular gland removal has been carried out successfully in other studies with no detrimental effects to survival, and has been shown to prevent the production of QMP (Maisonnasse, Alaux, *et al*., 2010).

To conclude, I present evidence that QMP's effects on ovary activity are lost at high doses, consistent with the hypothesis that caste-specific differences in QMP exposure rates underlie the evasion of self-sterilisation in queens. Further study is required at earlier timepoints to capture the hormetic mechanisms preceding this phenotype, with a possible contender being over-stimulation of dopamine receptors by HVA at high QMP doses. It is acknowledged that this proposed mechanism of self-sterilisation evasion rests heavily on the assumed higher queen exposure to QMP, and verification of this would be essential going forward. In addition, manipulative experiments in queens involving the inhibition of QMP production via mandibular gland removal and subsequent assessment of reproductive physiology would be a useful means of testing the relationship between QMP exposure and fecundity in honey bee queens. This is an emerging research area, and further investigations into alternative mechanisms of caste-specific differences in QMP's repression would ultimately aid the reconciliation of the contradictions within the queen control hypothesis.

Chapter 6

Investigating the interactivity between QMP and imidacloprid

6.1 Introduction

Insects provide crop pollination services that are integral for food security, with almost half of the 115 leading global food commodities reliant on their pollination (Klein *et al*., 2007). Honey bees in particular are among the most economically important pollinators in agricultural systems (Gallai *et al*., 2009). Our reliance on the ecosystem services of honey bees has grown throughout the 21st century: Between 1961 and 2006, dependence on pollinators by the agriculture industry grew by an estimated 50% (Aizen & Harder, 2009). Despite the increased demand, honey bee populations are diminishing, with reports of unsustainably high colony losses from Canada, the US and Europe (Chauzat *et al*., 2016; Lee *et al*., 2015; van der Zee *et al*., 2012; vanEngelsdorp & Meixner, 2010).

Declines of honey bee populations, and other pollinators alike, have been attributed to a combination of environmental stressors with synergistic negative effects on pollinator health (reviewed by Potts *et al*., 2010). In honey bees, these include (but are not limited to): diseases and parasites (i.e. The parasitic mite Varroa destructor: Martin *et al*., 1998 and Nosema: (Genersch, 2010), land use change (Woodcock *et al*., 2017), weather and climate change (VanEngelsdorp *et al*., 2008), and pesticide use (Tsvetkov *et al*., 2017; Woodcock *et al*., 2017). Within these stressors, the widescale use of agrochemicals has been identified as a particular concern to honey bee colonies given their role as agricultural pollinators.

The application of pesticides is a controversial but ultimately necessary agricultural practice to prevent otherwise devastating losses from pest species (Sharma *et al*., 2019). Chemical substances developed to control herbivorous insect pest populations are frequently picked up by non-target pollinators such as honey bees, who come into contact with these insecticides through their foraging activity. Contaminated floral resources are frequently brought back to the hive, where they present a further

exposure risk to other colony members including young workers, the queen, and developing larvae (Rortais *et al*., 2005).

Among the most widely used pesticides of recent years are neonicotinoids, a class of neurotoxic insecticides including thiamethoxam, imidacloprid, and clothianidin. These pose a prominent risk to honey bees and other pollinators. Their application has grown rapidly since the early 2000s, replacing organophosphates and pyrethroids to become the most widely used pesticides worldwide as of 2013 (Casida & Durkin, 2013). In response to a flood of high-impact studies reporting negative effects on non-target beneficial insects (e.g. Gill *et al*., 2012; Henry *et al*., 2012; Whitehorn *et al*., 2012), neonicotinoids were banned by the EU in 2020, but may still be used in 'emergency' situations. In the UK, for instance, emergency approval has recently been granted for the use of neonicotinoids on sugar beet crops in 2022 (Barkham, 2022). Neonicotinoids have been shown to persist in the soil, thus even one-off use increases the exposure risk of non-target organisms for years to come (Reviewed by Pietrzak *et al*., 2020).

Neonicotinoids are applied to crops as a seed dressing which is taken up upon germination and distributed systemically throughout the plant, making them direct targets of herbivorous pests that attack crop tissue. They act as neurotoxins, mimicking the action of the biogenic amine acetylcholine (ACh) by selectively binding to post-synaptic nicotinic aetylcholine receptors (nAChRs) in the insect brain. Usually, synaptic activation is terminated by the AChE enzyme, which rapidly breaks down ACh. However, neonicotinoids are insensitive to the action of AChE so persist in the synapse. The resulting persistent activation of nAChRs leads to convulsions, paralysis and eventual death in target pest species (Casida & Durkin, 2013; Casida & Quistad, 2004). Through exposure to the trace amounts of these pesticides present in pollen and nectar (Dively & Kamel, 2012; Mullin *et al*., 2010), pollinators such as honey bees are at risk less from acute toxicity, but more from sub-lethal effects of chronic exposure.

The adverse effects of sub-lethal neonicotinoid exposure on honey bees are well-documented. For example, neonicotinoids have been associated with reduced foraging

activity (Schneider *et al*., 2012), impaired navigation (Fischer *et al*., 2014), homing-failure (Henry *et al*., 2012), interrupted motor function (Williamson *et al*., 2014), impaired learning and memory (Decourtye, Armengaud, *et al*., 2004), and decreased colony performance and productivity (Sandrock *et al*., 2014). Despite the research attention accrued by the behavioural effects of sub-lethal neonicotinoid exposure, very little is known about their molecular underpinnings (a pattern true for many pesticides – reviewed by Christen *et al*., 2018). In particular, although the acute neurotoxic effects of neonicotinoids on target species are well understood, there is a shortage of research into the effects of chronic sub-lethal exposure on the neuroendocrine system in relation to behavioural modulation.

In honey bees, the neuroendocrine system of workers is under the significant additional influence of QMP (i.e. Chapter three). QMP is a multi-functional pheromone that acts as both a releaser of rapid behavioural responses (Wyatt, 2003) and a primer for long-term changes in behaviour and reproductive physiology. As a releaser, QMP stimulates retinue response behaviour, or the attraction of workers to the queen (Slessor *et al*., 1988; Vergoz *et al*., 2009). This attraction to the queen (or more specifically, to QMP) ensures workers feed and groom the queen and distribute her pheromones throughout the hive (Slessor *et al*., 1988; Slessor *et al*., 2005). Primer functions of QMP include the inhibition of ovary development (Hoover *et al*., 2003) and the slowed behavioural transition from nurse to forager (Pankiw *et al*., 1998). These functions of QMP are integral for colony productivity, ensuring queen-worker cooperation by suppressing individual worker reproduction (refer Section 1.2, General Introduction), as well as maintaining the division of labour and temporal polyethism in the hive (Pankiw *et al*., 1998).

QMP exerts at least some of these varied effects on workers through modulation of neuroendocrine signalling pathways. In particular, biogenic amines have been implicated as key messengers of QMP's presence, inciting downstream effects on physiology and behaviour as discussed in Chapter three and Chapter four. Biogenic amines are a highly conserved class of molecules which function as neuromodulators,

neurohormones and neurotransmitters (Evans, 1980), with additional evidence also implicating octopamine and dopamine as circulatory neurohormones (Chapter three). Although it has not been studied in honey bees, neonicotinoids disrupt biogenic amine signalling in other insect species via nAChR mediated dopamine release. The neonicotinoid imidacloprid induced dopamine release in larval *D. melanogaster* central nervous systems (Pyakurel *et al*., 2018), and long-term exposure caused a reduction in brain dopamine levels (hypothesised to be due to blockade of nAChR following prolonged exposure; Janner *et al*., 2021). Octopamine release is also mediated by nAChR stimulation in *D. melanogaster* (Fuenzalida-Uribe *et al*., 2013), thus octopamine levels may also be altered by neonicotinoids. Disruptions to neural octopaminergic signalling have been proposed as an explanatory mechanism behind some of the behavioural effects of neonicotinoids in honey bees (Farooqui, 2013), though to my knowledge this has not been empirically tested. Considering the importance of QMP's modulations of dopamine and octopamine to worker activities and colony harmony, our lack of understanding into how neonicotinoids affect these biogenic amines in honey bees represents a crucial gap in our knowledge.

Here, I test the hypothesis that neonicotinoids disrupt worker responses to QMP by interfering with octopamine and dopamine signalling, both of which are A. implicated as messengers of QMP's effects on workers and B. likely to be affected by neonicotinoid exposure. This is split into two sub-hypotheses (H1 and H2):

H1) Neonicotinoids disrupt worker behavioural and physiological responses to QMP

H2) disruptions to dopamine and octopamine signalling are the mechanisms underlying H1.

To test H1, worker responses to QMP were measured in bees undergoing a range of chronic imidacloprid exposure regimes. Three major endpoints were selected to assess worker responses to QMP, testing QMP's primer (long-term physiological and behavioural change) and releaser (short-term behavioural change) functions. The releaser function of QMP was tested by assessing the degree of attraction of workers to

the queen, or their 'retinue response' behaviour. Primer functions were tested by examining A. the locomotor activity of workers as a proxy for the behavioural transition to foraging, and B. ovary activity levels as a measure of QMP's induction of sterility.

To test H2, dopamine and octopamine levels were measured in workers following chronic imidacloprid regimes in combination with chronic QMP exposure. These biogenic amines are thought to act as both neurohormones (i.e. In the brain) and circulatory hormones (i.e. Interacting directly with peripheral tissues) to exert effects on workers (Chapter three). To understand their underlying role in coordinating worker responses to QMP and imidacloprid, octopamine and dopamine levels were measured in both the brain and hemolymph of workers following imidacloprid and QMP treatments.

To my knowledge, this is the first study investigating the effects of neonicotinoids on adult worker responses to QMP. In particular, the mutual manipulation of aminergic signalling pathways by both QMP and neonicotinoids have not previously been considered alongside one another. This is therefore a novel hypothesis, and this chapter sheds light on additional underlying mechanisms of the known negative effects of neonicotinoids on honey bee colony health and productivity.

6.2 Methods

6.2.1 Microcolony set-up and experiment overview

A. mellifera laboratory microcolonies were setup from source hives and maintained in cages as described in Section 2.1.1. Microcolonies each contained 100 workers and were exposed to imidacloprid at varying concentrations. To observe the interactive effects of imidacloprid with QMP, each imidacloprid treatment was tested both with and without continual QMP exposure. Two discrete experiments were conducted to address H1) that neonicotinoids disrupt worker behavioural and physiological responses to QMP and H2) that disruptions to dopamine and octopamine signalling are the mechanisms underlying H1. An overview of each experiment is detailed bellow.

Experiment one – behavioural and reproductive responses to QMP (H1)

The aim of experiment one was to assess the impacts of imidacloprid on QMP's modulation of worker behaviour and reproduction. Attraction of workers undergoing different imidacloprid and QMP treatment regimes to QMP was tested on day four in the retinue response assay, and locomotor activity levels were tested on day five as a proxy for foraging behaviour. In each assay, 10 randomly selected bees were tested from each cage. Bees used in behavioural assays were discarded following testing and not returned to their cage. On day 10, the ovaries of all remaining bees in each cage were dissected and imaged for later analysis of ovary activity levels as described previously (Section 2.2). Each experimental cage had three independent replicates (i.e. for behavioural assays n=30 bees in total). Within each replicate, all treatment cages were set up within two days of each other. All three replicates of experiment one were carried out between July – August 2021 under my supervision by two Biodiversity and Conservation MSc students, Jessica Bouwer and Emily Ross.

Experiment two – biochemical responses to QMP (H2)

Experiment two aimed to investigate the underlying mechanisms of experiment one by measuring honey bee internal dopamine and octopamine levels following each imidacloprid and QMP exposure regime. Cages were maintained as in experiment one. Whole brains and hemolymph samples were collected from 10 bees per cage on day 10 for future analysis of biogenic amine content using high-performance liquid chromatography with fluorescent detection (HPLC-FLD) as described (Section 2.3). Ovary activity levels and locomotor activity were measured in a subset of bees as a means of validating the consistency of QMP and neonicotinoid treatments between experiments one and two (see Appendix I, Figure I.1 for ovary activity in experiment two). Three replicates of each cage were carried out in September 2021 within three days of one another.

6.2.2 Imidacloprid and QMP treatments

Cages underwent chronic pesticide exposure regimes by spiking their water solutions with imidacloprid, while control cages received just water. The chronic lethal imidacloprid dose for honey bees (LC50), or the expected concentration associated with death of 50% of bees feeding on it, is estimated as 1,760 parts per billion (ppb) (Cresswell, 2011). A preliminary assessment of survival rates at a range of imidacloprid concentrations was carried out to verify pesticide efficacy and determine sublethal dosages within the laboratory setup. Cages of 100 workers were setup as described above and mortality rates recorded at 0, 100, 250, 500 and 1,000 ppb imidacloprid exposure in June-July 2019. In June-July 2021, survival was monitored at 0, 1 and 5 ppb imidacloprid. Determination of sub-lethal dosages was carried out in the absence of QMP, as is comparable with previous assessments of pesticide lethality. Imidacloprid was found to pose little risk to mortality under chronic exposure regimes of 100 ppb and bellow in our laboratory setup (see Appendix J, Figure J.1). Doses of 1 ppb and 5 ppb were selected for assessment, as these are within the realistic range in which honey bees are likely to come into contact with imidacloprid in the field (estimated to be between 0.7-10 ppb; Cresswell, 2011).

Cages therefore received one of six treatments: 0 ppb (control), 1 ppb or 5 ppb chronic imidacloprid exposure, each tested in both the presence (QMP+) or absence (QMP-) of continual QMP exposure. QMP treatments were administered as described previously (Section 2.1.2). Imidacloprid solutions were supplied ad libitum and replaced every three days. Mean solution intake per bee per day was recorded for each cage by weighing the solution tube daily.

6.2.3 Retinue response assay

Responses to QMP were tested on four day old workers, as this is the age at which workers are typically of nursing age and show the greatest attraction to QMP (Vergoz *et al*., 2009). QMP attraction, or ‘retinue response’, was assessed using a Petri dish divided

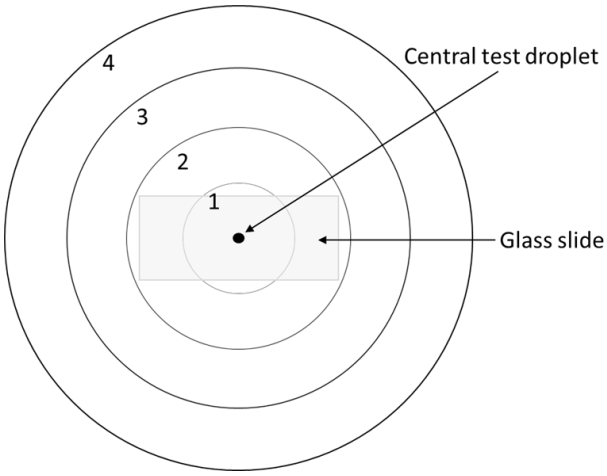

Figure 6.1 Diagram of the retinue response assay arena. A petri dish divided into four concentric sectors was used as the behavioural arena for the retinue response assay following the methodology used by Vergoz *et al*., (2009). Sectors were numbered to calculate area occupancy scores.

into four concentric sectors as a behavioural arena (Figure 6.1). Petri dish lids were modified to consist of a mesh layer to allow odours to dissipate and not fill the whole dish homogenously. The responses of bees to a 10 µL droplet of 0.01 QE/µL synthetic QMP vs a 10 µL ethanol droplet placed in the centre of the arena were compared. Bee responses were tested to ethanol droplets as a comparative control to account for individual differences in locomotor activity and inquisitiveness between bees. The order of droplet presentation was randomised for each bee and petri dishes were cleaned with 70% ethanol and aired between every trial. Trials were carried out under red light, as bees cannot see red light thus mimicking the darkness of the hive (Kimura *et al*., 2014). Trials were recorded two at a time from a birds-eye view of the arena using a GoPro camera and tripod for later analysis of the attraction of each bee to the central droplet (Figure 6.2). Videos were three minutes in duration following an initial one minute acclimatization period which began immediately after the bee was placed in the arena.

Attraction to each test droplet was assessed from video footage using the methodology described by (Vergoz *et al*., 2009). The sector in which the bee was located was recorded every 10 seconds. Sectors were numbered from one to four, with one being the central sector increasing to four in the outermost sector (Figure 6.1). A bee was classified as

Figure 6.2 Photo of the retinue response assay filming set-up. A GoPro was positioned in a birds eye view above two behavioural arenas to enable simultaneous recording of two trials at once. Though the setup is pictured in normal lighting, assays were performed under red light. Photo was taken by Emily Ross and used with permission.

being in a sector when over half of its body occupied it, or when the body was positioned equally between two sectors, the sector occupied by the head was recorded. The area occupancy score was calculated by multiplying each sector by the number of times the bee was recorded in that sector, then taking the sum for each sector. For example, if a bee occupied sector one once, sector two five times, sector three five times and sector four seven times, the area occupancy score would be:

(1 x 1) + (2 x 5) + (3 x 5) + (4 x 7) = 54

A bee spending most of its time in the central sectors will have a lower area occupancy score than a bee spending more time in the outermost sectors. As sector one contains the central droplet, a lower area occupancy score corresponds to a greater attraction to the test droplet.

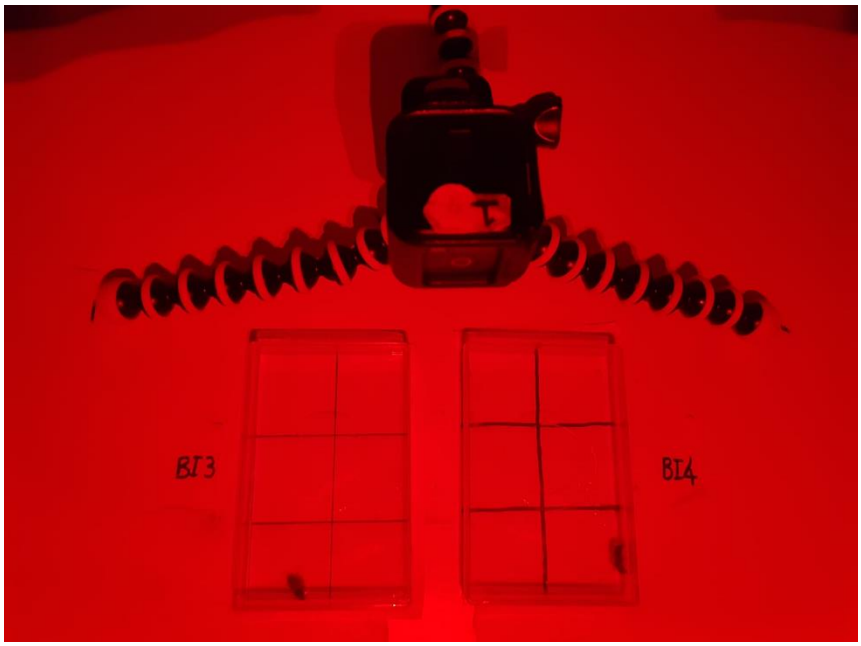

Figure 6.3 Photo of the locomotor activity assay filming set-up. A GoPro was positioned in a birds eye view above two behavioural arenas to enable simultaneous recording of two trials at once. Trials were recorded under red light, as pictured.

6.2.4 Locomotor activity assay

The locomotion assay was used to assess activity levels across treatment groups, as high activity levels are associated with foraging performance (Klein *et al*., 2019). Locomotor activity may therefore be seen as a very rudimentary proxy for the behavioural transition from nurse to forager, similarly to task allocation and personality in ants (Dr Victoria Norman, personal communication). The locomotor assay used was modified from Williams *et al*., (2020). The behavioural arena was a rectangular petri dish (12cm x 8 cm x 2 cm) divided into 6 equal square sections (Figure 6.3). The number of times a bee crossed a line from one section into another was recorded. A cross was only recorded if the bee's entire body crossed from one section into an adjacent section. Crosses made diagonally between sections were recorded as one line cross. As above, trials were carried out under red light and recorded for later analysis of video footage, and the trial duration was 3-minutes following an initial 1-minute acclimatization period. Bees were tested in blocks of ten at a time randomised across treatment groups. 'Assay block' was

recorded for each bee and included as a random effect in data analysis (described in section 6.2.5).

6.2.5 Data analysis

All statistical analyses were carried out using R. Details of packages used for analysis are found in section 2.4. For all analyses, imidacloprid dose was considered an ordinal variable, as the number of doses tested was insufficient to be considered a continual variable.

To assess whether imidacloprid and QMP treatments caused confounding effects on food and water consumption, analyses were performed on the effects of QMP and imidacloprid on food and water intake. Linear mixed effects models (LMMs) were fitted with mean food or water consumed per bee per day as the response variables and QMP, imidacloprid dose and their interaction were as fixed effects. To account for repeated measures, day was included as a random effect and nested within replicate. Visual inspection of residual plots showed no obvious deviations from normality or homoscedasticity. Statistical significance of fixed effects was obtained by comparing likelihood ratios of the maximal model to the model without the fixed effect of interest. *Post-hoc* testing was carried out on the maximal model by computing estimated marginal means. P-values were Tukey-adjusted to control for multiple testing.

The effects of QMP and imidacloprid dose on survival were tested using Cox proportional hazards models (CPH) with mixed effects. The fixed effects were QMP, imidacloprid and their interaction, with replicate included a random effect. The assumption of proportional hazards was verified for the model as a whole by correlating scaled Schoenfield residuals with time to test for independence. This model was used to predict Hazard Ratios (HR) and confidence intervals for different treatment combinations. HRs are presented as HR (± 95% confidence interval). P-values for all pairwise comparisons between treatments were obtained from the maximal model by computing estimated marginal means. Multiple testing was accounted for with Tukey-adjustment of P-values.

Area occupancy scores of each bee in the presence of the control droplet vs QMP droplet were compared using paired T-tests. Paired T-tests were carried out for every QMP/imidacloprid treatment combination to assess in which groups preferences for the QMP test droplet were seen. Effect sizes (d) were calculated by dividing the mean difference by the standard deviation of the difference of paired samples (Cohen's-d formula). Normality of differences between paired samples was confirmed through visual assessment of histogram plots.

Locomotor activity was assessed by LMMs on the number of line crosses with QMP presence, imidacloprid dose and their interaction as fixed effects. Replicate and assay block were included as random effects, with by-replicate and by-assay block random slopes for the effect of all fixed effects. Visual inspection of residual plots showed no obvious deviations from normality or homoscedasticity.

P-values for individual fixed effects and their interaction were obtained by comparing the likelihood ratio of the maximal model to that of the model without the fixed effect of interest (Bates *et al*. 2015). *Post-hoc* pairwise testing was carried out on the maximal model using estimated marginal means. P-values were Tukey-adjusted to control for multiple testing.

Ovary scores in each treatment group were analysed by fitting cumulative link mixed models (CLMMs) with Laplace approximation. QMP presence, imidacloprid dose and their interaction were included as fixed effects, and replicate was included as a random effect. Statistical significance of fixed effects was assessed by comparing the likelihood ratio of the maximal model to that of the model without the fixed effect of interest.

Analysis of brain and hemolymph octopamine and dopamine levels was carried out by fitting GLMMS. QMP, imidacloprid dose and their interaction were included as fixed effects and replicate and HPLC batch were included as random effects with random intercepts. LMMs were initially fitted and residual distributions were tested by visual examination of qqplots and histograms. In all cases these were not normally distributed so GLMMs were instead fitted. Histograms of the raw data were used to decide model

family and link function. For brain octopamine and dopamine, GLMMs with a gamma distribution and an inverse link function were fitted. For hemolymph octopamine and dopamine a log link function was used. Statistical significance of fixed effects and their interaction was tested by comparing the likelihood ratio of the maximal model to that of the model without the fixed effect of interest (Bates *et al*. 2015). If fixed effects were statistically significant, *post-hoc* pairwise testing was carried out on the maximal model using estimated marginal means. P-values were Tukey-adjusted to control for multiple testing.

6.3 Results

This study aimed to test the hypothesis that exposure to neonicotinoid pesticides disrupts honey bee worker responses to QMP (H1). Through the assessment of a series of endpoints testing established behavioural and physiological effects of QMP on workers, interactive effects between imidacloprid and QMP are studied here for the first time. The hypothesis that disruptions to aminergic signalling underly these effects was also tested by quantifying dopamine and octopamine levels in key worker tissues (H2).

6.3.1 Effects of imidacloprid and QMP treatments on food and water intake

Food intake was measured daily for each cage to assess whether there were any confounding effects of QMP and imidacloprid treatments on nutritional status that could impact experimental results. Water intake (or imidacloprid solution in pesticide treatments) was also recorded daily for each cage, to verify the administration of treatment solutions and estimate imidacloprid dosages received. As experiment one (assessment of ovary activity and behaviour – H1) and experiment two (assessment of internal biogenic amine levels – H2) were conducted at separate times, these are considered two discrete experiments and food and water consumption data from each are reported independently of one another.

in experiment one, food intake did not differ between treatment groups (Figure 6.4). Comparisons of LMMs with QMP, imidacloprid and their interaction as fixed effects

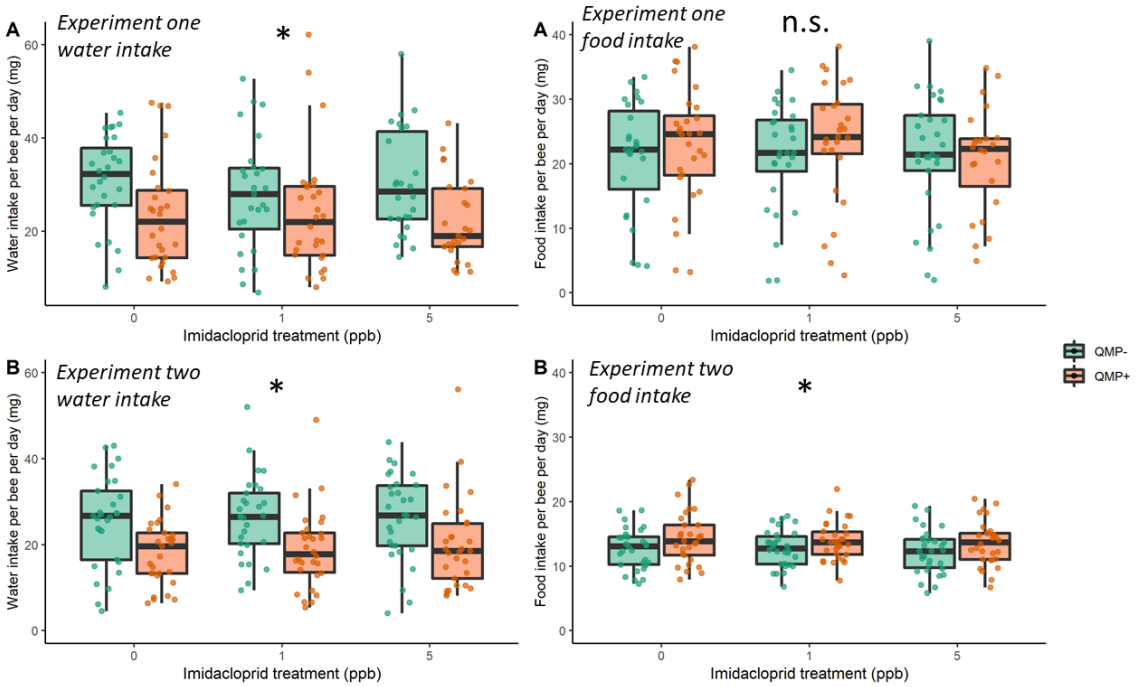

Figure 6.4 Food and water intake of workers treated with QMP and imidacloprid. Workers were housed with QMP (QMP+, orange) or solvent control (QMP-, green) and treated with 0, 1 or 5 ppb imidacloprid in **A.** Experiment 1 and **B.** Experiment 2. Food and water consumption was recorded daily for each treatment cage (n = 10 days), and each treatment had three independent replicates (n = 30 total). Pairwise *post-hoc* comparisons were carried out where LMM model comparisons revealed statistical significance of imidacloprid or QMP (denoted by "*"). Due to the large number of pairwise comparisons, these are not presented on the figure and can instead be found in Appendix K, Table K.1. "n.s" represents statistical non-significance.

showed that neither QMP, Imidacloprid, nor their interaction had statistically significant effects on food consumption (LMMs: QMP: $\chi2 = 3.02$, df = 3, 161, $p = 0.39$; Imidacloprid: $\chi2 = 0.88$, df = 4, 160, $p = 0.93$; Interaction: $\chi2 = 0.17$, df = 2, 162, $p = 0.92$), indicating that nutritional intake does not contribute to any treatment-level differences in ovary activity, locomotor activity or retinue response results. In experiment two, in which biogenic amines were quantified, food intake was slightly lowered by imidacloprid treatment (Figure 6.4; LMMs: $\chi2 = 10.26$, df = 4, 174, $p < 0.05$). Additionally, bees consumed more food when exposed to QMP (LMMs: $\chi2 = 44.75$, df = 3, 175, $p < 0.001$), though there was no evidence for an interaction effect of imidacloprid and QMP (LMMs:

$\chi 2 = 2.18$, df = 2, 176, p = 0.36). A full table of all pairwise comparisons revealed by *post-hoc* tests is displayed in Appendix K, Table K.1.

Across both experiment one and experiment two, bees maintained in the presence of QMP consumed significantly less water than those maintained without QMP (Figure 6.4; LMMs; Experiment one: $\chi 2 = 23.92$, df = 3, 158, $p < 0.001$; Experiment two: $\chi 2 = 50.18$, df = 3, 172, $p < 0.001$). It should therefore be noted that as imidacloprid was administered in water solutions, pesticide dosages received will be slightly elevated in QMP- treatment cages. See Appendix K, Table K.1 for *post-hoc* pairwise comparisons between treatments calculated using estimated marginal means. Imidacloprid treatment had no effect on water consumption in experiment one and two (LMMs; Experiment one: $\chi 2 = 1.86$, df = 4, 157, p = 0.76; Experiment two: $\chi 2 = 2.03$, df = 4, 171, p = 0.73). There was also no evidence to support an interaction effect between QMP and imidacloprid on water intake in either experiment (LMMs; Experiment one: $\chi 2 = 1.43$, df = 2, 159, p = 0.49; Experiment two: $\chi 2 = 0.24$, df = 2, 173, p = 0.89), indicating that there were neither repulsive nor addictive effects of pesticide solutions at the concentrations given.

6.3.2 Ovary activity is repressed by synthetic QMP regardless of imidacloprid treatment

To test whether imidacloprid treatment disrupts QMP's ability to inhibit worker reproduction, ovary activity levels were assessed in workers housed with or without QMP (QMP+/ QMP-) following different chronic imidacloprid treatment regimes. In this laboratory setting, the efficacy of synthetic QMP is demonstrated by its repression of worker ovary activity in imidacloprid control groups (Figure 6.5). When comparing 0 ppb imidacloprid controls, 24.22% of 10-day old workers housed without QMP had ovaries containing mature oocytes (ovary score of 3 – highest degree of ovary activity; Figure 2.2), while this number was reduced to 1.74% in the presence of QMP (Figure 6.5).

To test whether imidacloprid treatment affected QMP's regulation of worker reproduction (H1), CLMMs were fitted with the maximal model containing imidacloprid,

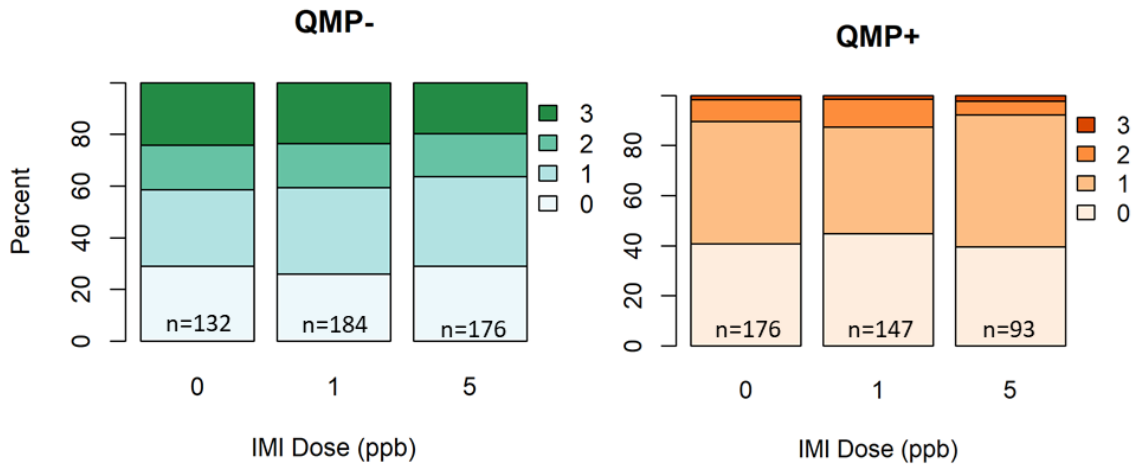

Figure 6.5 Ovary activity is repressed by QMP regardless of imidacloprid dosage. Ovary activity levels of 10-day old worker honey bees maintained in the presence of QMP (**QMP+,** shades of orange) or solvent control (**QMP-**, shades of green) following 0 ppb (control), 1 ppb and 5 ppb imidacloprid (IMI) treatment. Ovary activity is presented as the percentage of bees within each treatment group with each ovary score. Ovary scores range from 0-3 (see Figure 2.2), where a higher score (darker shade) relates to a higher degree of ovary development. All treatment cages began with 100 bees, and all remaining bees on day 10 (after deaths and removal for behavioural assays) were dissected. N values are displayed at the base of each bar. Data shown is from experiment 1 and is pooled over three replicates of each treatment. Exceptions are QMP+ 5 ppb, where all bees in replicate three died prior to ovary dissection, and QMP- 0 ppb, where a technological failure caused the loss of all ovaries imaged from replicate one.

QMP and their interaction as fixed effects, and reduced models containing all but the fixed effects of interest. As discussed above, model comparisons showed QMP's repression of ovary activity to be statistically significant (CLMM: $\chi2 = 73.91$, d.f = 3, 885, $p < 0.0001$), which is consistent with previous studies using this laboratory system (Duncan *et al*., 2016). Within this setting, QMP's effects on ovary development were unaltered by pesticide treatment; Imidacloprid had no effect on ovary activity levels at any dose either on its own (CLMM: $\chi2 = 1.35$, d.f = 4, 884, $p = 0.85$) or through interactions with QMP (CLMM: $\chi2 = 1.094913$, d.f = 2, 886, $p = 0.58$), indicating that the primer effects of QMP on worker ovary development are unaffected by neonicotinoid exposure.

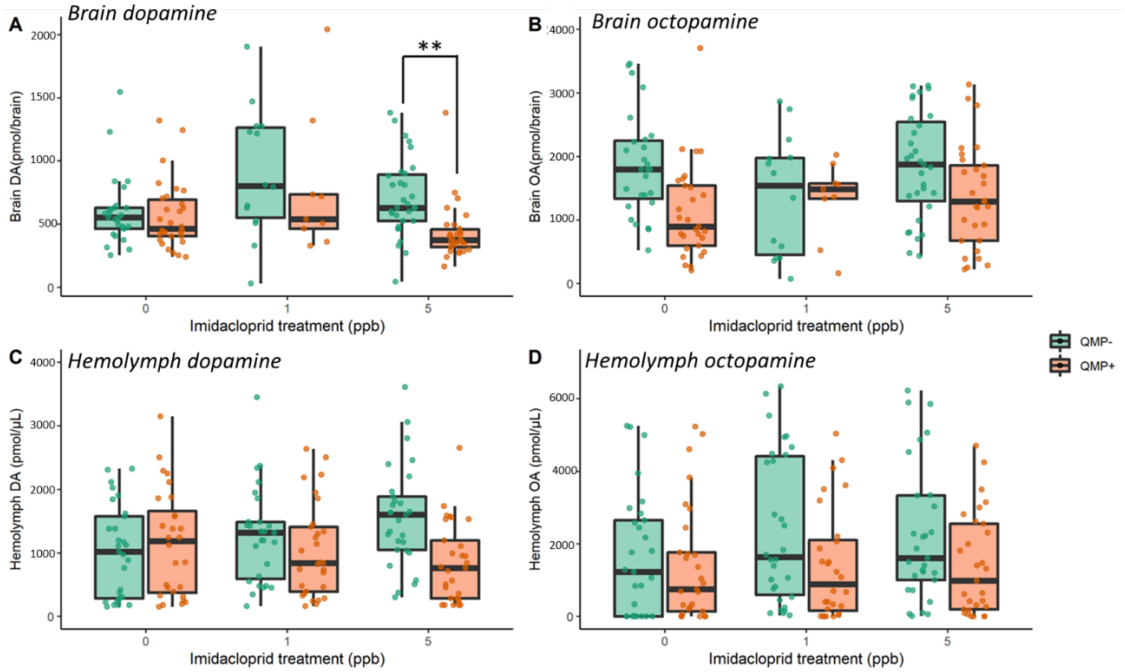

Figure 6.6 Effects of QMP and imidacloprid treatments on biogenic amine levels. Levels of dopamine (DA) and octopamine (OA) in tissues of 10-day old laboratory-raised worker honeybees following different doses of IMI treatment, where workers were maintained in experimental cages of 100 bees and received 0.1 QE of QMP (+QMP) or solvent control (-QMP) every 24 hours. HPLC-FLD was used to quantify levels of DA and OA from samples extracted from 10 randomly selected bees per treatment for three experimental cage replicates (n=30 per treatment total). Statistical significance between treatments in *post-hoc* tests at the level of $p < 0.01$ is highlighted by (**). Full table of *post-hoc* pairwise tests is found in Appendix K, Table K.2. **(A)** Brain dopamine levels were lower in +QMP bees relative to –QMP. This was statistically significant only for bees receiving 5ppb IMI. **(B)** Brain OA levels were lower +QMP, but not significantly so, and are unchanged by IMI treatment. **(C, D)** DA and OA were detected in worker hemolymph but were unaffected by either IMI treatment or QMP exposure.

6.3.3 Imidacloprid interacts with QMP to lower brain dopamine levels

To test whether imidacloprid interferes with QMP's modulation of biogenic amines (H2), dopamine and octopamine were quantified in the brains and hemolymph of workers following different QMP and imidacloprid treatment regimes (Figure 6.6). For each biogenic amine and each tissue, GLMMs were fitted with QMP, imidacloprid and their interaction as fixed effects. As is consistent with in-hive studies, synthetic QMP was associated with a statistically significant reduction of both dopamine and octopamine levels in 10-day old worker brains (GLMMs: Brain dopamine: $\chi 2 = 15.78$, d.f = 3, 138, $p <$

0.01; Brain octopamine: $\chi2 = 1.21$, d.f = 3, 137, $p < 0.05$). Pairwise *post-hoc* testing using estimated marginal means revealed QMP's reduction of brain dopamine levels had the largest effect size and was statistically significant only in combination with 5 ppb imidacloprid treatment ($Z = -3.84$, $p < 0.01$; for full table of *post-hoc* comparisons see Appendix K, Table K.2). Additionally, GLMM model comparisons showed that although imidacloprid alone had no measurable effect on brain dopamine levels (GLMM: $\chi2 = 8.26$, d.f = 4, 137, $p = 0.83$), there was a statistically significant interaction effect of imidacloprid on QMP (GLMM: $\chi2 = 7.51$, d.f = 2, 139, $p < 0.05$). Put simply, 5 ppb imidacloprid treatment enhanced the depressive effects of QMP on brain dopamine levels in workers, a phenomenon which has not previously been demonstrated.

In contrast, there was no evidence for any effects of imidacloprid treatment on brain octopamine levels (GLMM: $\chi2 = 2.94$, d.f = 4, 136, $p = 0.57$), nor for interaction effects with QMP (GLMM: $\chi2 = 1.48$, d.f = 2, 138, $p = 0.48$). In the hemolymph, QMP treatment was associated with a slight reduction of octopamine levels (as has been shown in the hive – Chapter three), although this was not statistically significant (GLMM: $\chi2 = 3.24$, d.f = 3, 175, $p = 0.35$). Imidacloprid also had no measurable effect on hemolymph octopamine levels, either alone (GLMM: $\chi2 = 3.13$, d.f = 4, 174, $p = 0.54$) or through interactions with QMP (GLMM: $\chi2 = 2.91$, d.f = 2, 176, $p = 0.23$). Hemolymph dopamine levels, which have not previously been found to be altered by QMP in 10-day old laboratory workers (Chapter three), showed a similar trend to brain dopamine levels whereby QMP's depression was exacerbated with increasing imidacloprid dose. However, no significant effects on hemolymph dopamine levels were caused by QMP, imidacloprid, or their interaction effect (QMP: $\chi2 = 2.07$, d.f = 3, 175, $p = 0.56$; Imidacloprid: $\chi2 = 1.68$, d.f = 4, 174, $p = 0.79$; Interaction: $\chi2 = 1.24$, d.f = 2, 176, $p = 0.54$).

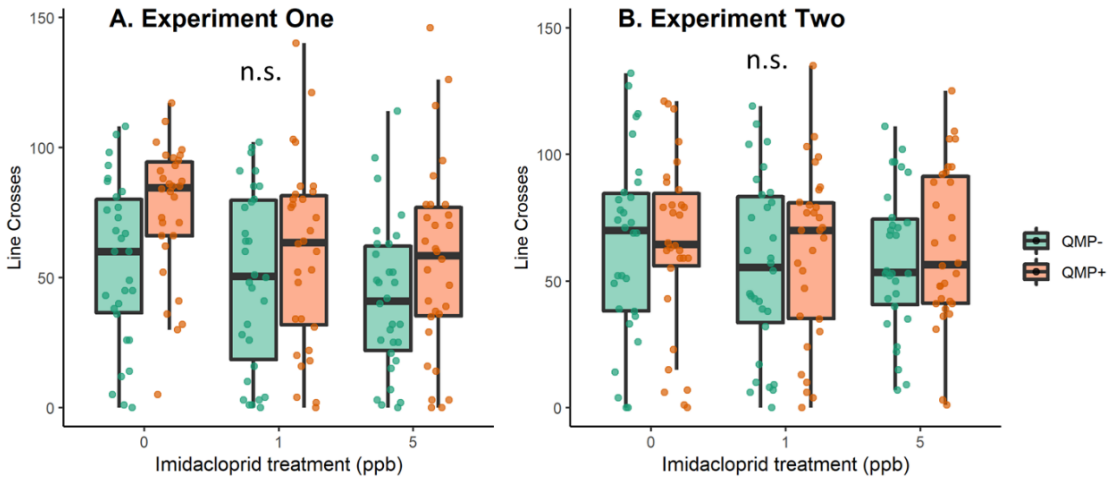

Figure 6.7 Locomotor activity of QMP and imidacloprid treated workers. Locomotor activity of five-day old bees undergoing different imidacloprid treatments in the presence (**QMP+** orange boxplots) and absence (**QMP-** green boxplots) of QMP in **A.** Experiment one and **B.** Experiment two. N = 30 for each imidacloprid treatment (three pooled replicates where 10 bees were assayed each replicate). Locomotor activity is measured by the number of line crosses made by a bee over the three-minute assay duration. Overall statistical non-significance from LMM model comparisons is denoted by "n.s."

6.3.4 QMP and imidacloprid did not affect the locomotor activity of five-day old workers

Locomotor activity was studied as a proxy for foraging behaviour. There was no evidence for any effects of QMP, imidacloprid, or their interaction on locomotor activity in five-day old workers (Figure 6.7). Comparisons of LMMs showed that QMP and imidacloprid had no statistically significant impacts on locomotor activity in experiment one (LMMs; QMP: $\chi2 = 4.02$, d.f = 3, 176, p = 0.26; IMI: $\chi2 = 3.83$, d.f = 4, 175, p = 0.43; Interaction: $\chi2 = 1.70$, d.f = 2, 177, p = 0.43). The locomotor activity assay was repeated in experiment two to verify consistent behavioural responses to imidacloprid and QMP treatments across experiments for validation of biogenic amine data. Consistently with experiment one, no effects on locomotor activity were found in response to QMP treatment, imidacloprid treatment or their interaction effects (LMMs; QMP: $\chi2 = 0.56$, d.f = 3, 176, p = 0.91; IMI: $\chi2 = 1.04$, d.f = 4, 175, p = 0.90; Interaction: $\chi2 = 0.29$, d.f = 2, 177, p = 0.86; figure 7). However, there are likely limitations to this assay's efficacy as a proxy for forager behaviour, as discussed in section 6.4.

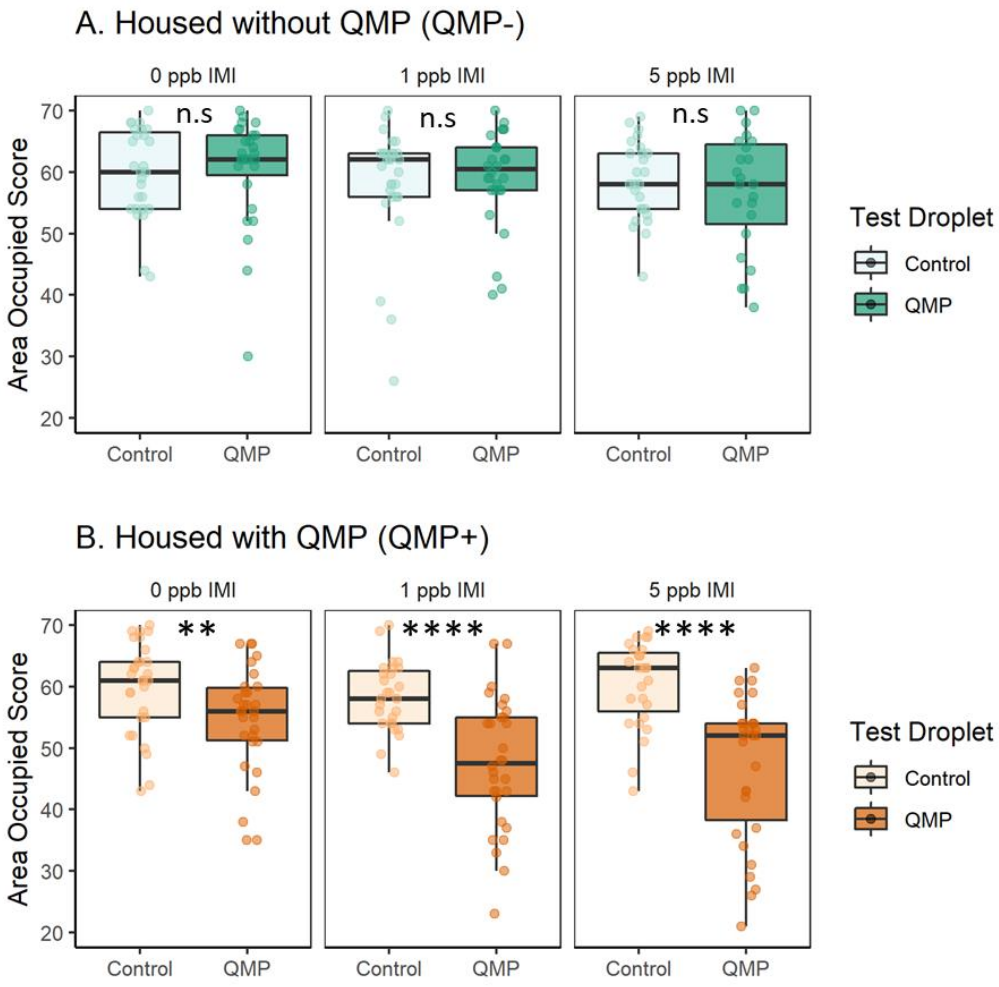

Figure 6.8 Attraction of workers to QMP Responses of imidacloprid (IMI)-treated four-day old worker honeybees to a test droplet of QMP (darker shaded boxes) vs a control droplet of ethanol (lighter shaded boxes) during a three-minute assay period. Workers were housed in cages with continual exposure to **A.** solvent control (**QMP-,** shades of green) or **B.** QMP (**QMP+,** shades of orange) prior to testing. Ten randomly selected bees were tested in each treatment group for three cage replicates (n=30 per treatment total). Relative attraction to test droplets is measured by area occupied score, where a lower score relates to a greater preference. Statistical significance between QMP vs control droplet areas at the levels of $p<0.01$** and 0.0001**** is highlighted by the corresponding numbers of asterisks. Statistical non-significance is denoted by "n.s". Bees that have had no prior exposure to QMP **(QMP-)** show no preference to QMP test droplets over control test droplets, and this is unaltered by IMI treatment. Bees that had seen continual exposure to QMP since adult emergence **(QMP+)** showed significantly increased attraction to QMP test droplets relative to control test droplets, and both the magnitude and statistical significance of this difference increased with IMI treatment.

6.3.5 Imidacloprid increases worker attraction to QMP

The attraction of workers to a test droplet of QMP vs ethanol controls was compared in four-day old bees that had been housed with or without QMP following different imidacloprid exposure regimes (Figure 6.8). “Area occupancy score” is the metric used to measure attraction to the test droplet, with a lower score relating to a higher degree of attraction. For workers that had been exposed to QMP since adult emergence (QMP+), increasing imidacloprid dosages were associated with an increase in the strength of attraction to QMP. In QMP+ 0 ppb imidacloprid controls, the test droplet received had a moderate effect on area occupancy scores, with scores being lower for QMP droplets than ethanol control droplets (Paired T-test: $T = 2.77$, $df = 29$, $p < 0.001$, $d = 0.51$, effect size moderate). QMP+ bees treated with 1 ppb imidacloprid showed a stronger response to QMP, as demonstrated by the larger effect size of the test droplet on area occupancy scores (Paired T-test: $T = 4.77$, $df = 29$, $p<0.0001$, $d=0.87$, effect size large). Interestingly, the highest degree of QMP attraction was seen in QMP+ bees receiving 5 ppb imidacloprid, where the effect size of test droplet on area occupancy was greater than in any other treatment group (Paired T-test: $T = 6.14$, $df = 29$, $p<0.0001$, $d=1.12$, effect size large). In short, the attraction of QMP+ workers to QMP became stronger with increasing imidacloprid exposure.

Consistent with published work (Vergoz *et al*., 2009), workers that had seen no prior exposure to QMP (QMP-) displayed no evidence of attraction to QMP. Paired T-tests showed there to be no statistically significant difference in area occupancy scores between QMP droplets and control droplets in –QMP 0 ppb imidacloprid controls (Paired T-test: $T = 0.29$, $df = 29$, $p=0.778$, $d=-0.05$, effect size negligible). Unlike those that had seen prior exposure to QMP, imidacloprid treatment did not alter the retinue responsiveness of QMP- bees, with 1 ppb and 5 ppb treatment groups also showing no preference for QMP droplets over control droplets (Paired T-test: 1 ppb and 5 ppb respectively: $T = -0.60, 0.03$, $df = 29,29$, $p=0.55, 0.98$, $d=-0.11, 0.01$, effect size negligible in both cases).

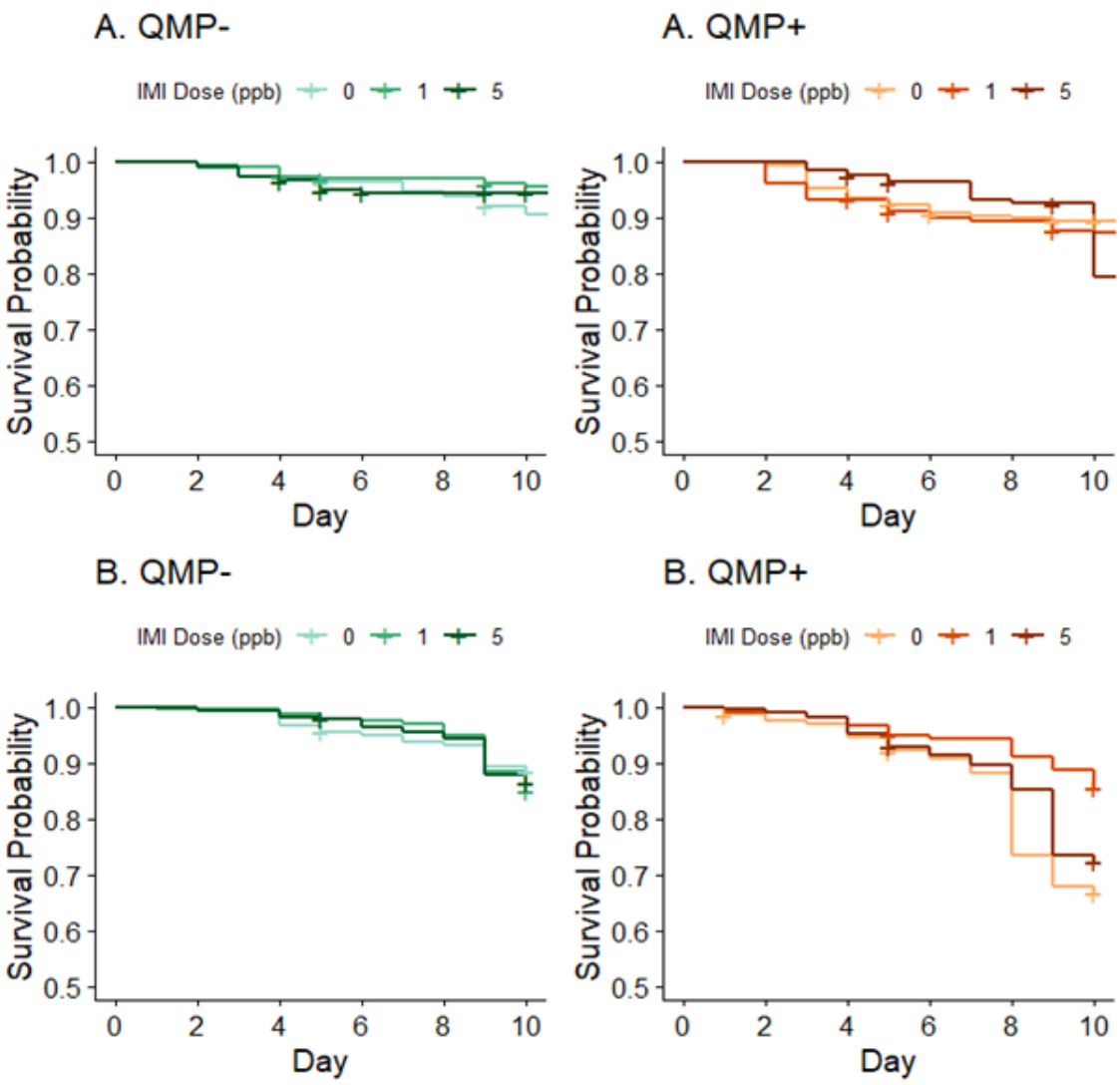

Figure 6.9 Effects of QMP and imidacloprid on survival Survival probabilities of cages of 100 workers maintained in the absence or presence of QMP receiving imidacloprid (IMI) solutions at concentrations of 0, 1 and 5 pbb. Increasing imidacloprid concentrations are denoted by darkening shades of green **(QMP-)** and orange **(QMP+).** Survival probabilities are shown for **A.** Experient 1 (July-August 2021) and **B.** Experiment 2 (September 2021). Data shown constitutes three independent replicates of each discrete experiment. One cage was removed from the dataset due to rapid unexpected death of all bees (Experiment 1, QMP+ 5PPB, replicate 3). Censoring events, for instance, the removal of bees from the cage for behavioural assays, are denoted by crosses. The Y axis has been truncated to begin at a survival probability of 0.5. For statistical significance of *post-hoc* pairwise comparisons of survival between different treatment groups, see Appendix K, Table K.3

6.3.6 QMP exposure may impact the lethality of imidacloprid

Analysis of survival rates between treatment groups was carried out using CPH models with mixed effects to assess whether the interactive effects of QMP and imidacloprid

affected mortality. As experiments one (assessment of ovary activity and behaviour – H1) and two (assessment of internal biogenic amine levels – H2) are considered two discrete experiments, survival data were analysed separately for each.

In cages maintained in the absence of QMP (as laboratory testing of imidacloprid lethality has previously been conducted), treatment with 1 and 5 ppb imidacloprid had no negative effects on survival (Figure 6.9) in both experiment one (1 ppb: HR = 0.47 ± 0.35 SE, z = -2.17, p = 0.06; 5 ppb: HR = 0.69 ± 0.31 SE, z = -1.17, p = 0.24) and experiment two (1 ppb: HR = 1.27 ± 0.24 SE, Z = 1.00, p = 0.32; 5 ppb: HR = 1.14 ± 0.24 SE; Z = 0.56; p= 0.58).

As a central facet of this study involved the determination of synergistic interactions between QMP and imidacloprid, the effect of QMP alone on survival in 0 ppb imidacloprid controls was analysed to assess whether QMP treatments caused inherent differences in survival. In experiment one, QMP treatment was not associated with any differences in survival in imidacloprid controls (HR = 1.25 ± 0.27 SE, z = 0.82, p = 0.41). In experiment two, exposure to QMP significantly increased the risk of mortality (HR = 3.34 ± 0.21 SE, z = 5.87, p < 0.001), although this trend was largely driven by one cage in which death rates were unusually high at 50%. It is suspected that this may have resulted from external factors, such as incidence of disease within the cage, as QMP treatment has generally not been associated with a reduction in survival throughout other chapters within this thesis.

Examining the combined effects of QMP and imidacloprid on survival rates revealed that in experiment one, exposure to QMP increased the lethality of imidacloprid. Pairwise *post-hoc* testing using estimated marginal means showed that bees receiving the same imidacloprid dose saw higher mortality rates when these were administered in combination with QMP. QMP exposure reduced the survival probabilities of bees at 1 ppb (z = - 4.54, p <0.0001) and 5 ppb imidacloprid treatment (z = -3.60, p <0.01). The same trend was observed in experiment two, though only at the 5 ppb dose (z = -4.09, <0.001), and the high mortality rates of QMP+ imidacloprid control limit the extent to

which synergistic effects of QMP and imidacloprid on mortality can be inferred For a complete table listing all *post-hoc* pairwise comparisons, see Appendix K, Table K.3.

6.4 Discussion

Produced by the queen, QMP is a major regulator of honey bee biology and in-hive dynamics (Hoover *et al*., 2003), promoting queen-worker cooperation and coordinating division of labour in the hive (Pankiw *et al*., 1998). Biogenic amines have been implicated as key messengers mediating the varied downstream behavioural and physiological effects of QMP (Beggs *et al*., 2007; Harris & Woodring, 1995; Sasaki & Nagao, 2001; Chapter three; Chapter four). There is also evidence, from other species, that neonicotinoids alter levels of the biogenic amines octopamine and dopamine (Fuenzalida-Uribe *et al*., 2013; Janner *et al*., 2021; Pyakurel *et al*., 2018), raising the possibility that these pesticides may interfere with worker interpretation of QMP's chemical communication. This chapter aimed to test two hypotheses: H1) that neonicotinoids interfere with established worker responses to QMP, and H2) that perturbations to aminergic signalling (namely octopamine and dopamine) play an underlying mechanistic role in H1. By investigating a series of well-documented worker responses to QMP, this chapter shows evidence for interactive effects between imidacloprid and QMP on worker behaviour, with speculative roles of biogenic amines underlying these behavioural alterations.

A notable finding was the increased attraction of workers to QMP following chronic imidacloprid treatment as measured by the retinue response assay. The attraction of young workers to QMP ensures workers feed and groom the queen and distribute her pheromones throughout the hive (Slessor *et al*., 1988; Slessor *et al*., 2005), and frequent contact with QMP slows the transition from nursing to foraging (Pankiw *et al*., 1998). Attraction to QMP is usually highest in younger workers, and the decline in attraction with age is associated with the transition to foraging (Pankiw *et al*., 1998; Vergoz *et al*., 2009). There is also a high degree of individual diversity in QMP responsiveness (Kocher *et al*., 2010; Walton & Toth, 2016), and these individual and temporal variations in

response thresholds facilitate effective division of labour in the hive (Walton *et al*., 2018). Therefore, the correct balance of worker attraction to QMP is integral for ensuring labour needs of the colony are met, through maintenance of appropriate numbers of nurses and foragers.

To my knowledge, the effect of adult worker exposure to neonicotinoids on QMP attraction has not previously been investigated. Larval exposure to a different class of pesticides, the insect growth disruptors (IGDs) pyriproxyfen and methoxyfenozide, has been shown to negatively affect worker attraction to QMP in adulthood (Litsey *et al*., 2021), in contrast with the increase in attraction seen with imidacloprid. These opposing phenotypes may be partially explained by the methodology used to measure QMP attraction. In both this study and Litsey *et al*., (2021), attraction was assessed through observations of worker responses to a strip of synthetic QMP. However, Litsey *et al*. (2021) made no comparative observations of workers to a control strip, meaning their reported loss of attraction could have arisen from reduced locomotor capabilities, lower energy reserves or diminished inquisitiveness to novel stimuli in pesticide-treated bees. Further, concentrations of pesticides used in the study were particularly high; larvae were treated with methoxyfenozide at concentrations 10-fold higher than have been reported in pollen stores of hives from the field (Rennich *et al*., 2014).

This being said, it may be expected that the effects on QMP attraction differ between classes of pesticides due to their range in target biochemical pathways. IGDs target pathways associated with insect growth and development (Pener & Dhadialla., 2012), with pyriproxyfen in particular being analogue of Juvenile Hormone (JH). JH functions as a gonadotropin in solitary and primitively eusocial insects, but a rewiring of this neuroendocrine network in worker honey bees has resulted in the loss of its reproductive function (Rodrigues & Flatt, 2016). JH instead plays a role in coordinating temporal polyethism (Robinson & Vargo, 1997), with a rise in JH associated with the transition to foraging (Schulz *et al*., 2002) and reduced attraction to QMP (Mcquillan *et al*., 2014). JH has been shown to reduce attraction of honey bee workers to QMP by lowering transcript levels of the octopamine receptor *AmOA1* in the antennae

(Mcquillan *et al*., 2014). Antennal expression levels of *AmOA1*, as well as the D2-like dopamine receptor *AmDop3*, positively correlate with QMP attraction, acting antagonistically with the D1-like receptor AmDop1 (Vergoz *et al*., 2009). Therefore, a reduction in worker QMP attraction caused by the JH analogue pyriproxyfen would be expected if this IGD has the same regulatory effects on antennal biogenic amine receptor expression as JH.

The potential mechanism behind the effects of imidacloprid on retinue response behaviour is more difficult to untangle. Here, it was hypothesised that neonicotinoids would disrupt biogenic amine levels in the brain, as has been demonstrated in *D. melanogaster* (Janner *et al*., 2021). Dopamine and octopamine both regulate JH degradation in *Drosophila* species (Gruntenko *et al*., 2005, 2007), though it is unclear whether this is true for honey bees also. Assuming biogenic amines also mediate JH levels in honey bees, neonicotinoids may reduce QMP attraction by decreasing JH titres indirectly through modulation of biogenic amine levels. However, further study is required on the relationship between dopamine and octopamine and JH titres in honey bees in order to test the occurrence and directionality of JH regulation. This could be achieved by manipulating internal biogenic amine levels in honey bees (i.e. using the methodologies discussed in Chapter four) followed by determination of JH titres using mass spectrometry (GC-MS) or HPLC-FLD with the addition of fluorescent JH labelling reagents (Rivera-Perez *et al*., 2012). Assessment of JH titres in honey bees following imidacloprid treatment would also clarify whether the increased attraction to QMP associated with imidacloprid exposure is underpinned by decreases in JH, with further measurements of antennal expression levels of the octopamine receptor *AmOA1* (which has been implicated in JH's mediations of QMP attraction as mentioned previously; Mcquillan *et al*., 2014).

The effects of neonicotinoids on JH titres have not been investigated in adult honey bees. However, one study has been carried out on honey bees at the larval stage, finding that exposure to thiamethoxam (another neonicotinoid pesticide) was associated with an increase in larval JH titres (Li *et al*., 2021). However, the effects of neonicotinoids on JH

titres are likely to differ between developmental stages, as ontogenetic differences in the effects of dopamine on JH have been established in a range of insect species. For example, in *D. melanogaster* females, dopamine has opposing effects on JH levels depending on developmental stage, causing an increase in young females and a decrease in sexually mature females (Gruntenko *et al*., 2005, 2012; Rauschenbach *et al*., 2011). If neonicotinoids alter JH levels via disruptions to dopamine's neurohormonal signalling in honey bees, they could thus plausibly elicit opposing effects on honey bee JH titres at larval and adult stages.

Support for the hypothesis that imidacloprid alters brain biogenic amine levels in honey bees (H2) was mixed. QMP's depression of octopamine levels was unaffected by imidacloprid, though brain dopamine levels were altered by imidacloprid exposure through an intriguing interactivity with QMP. In *D. melanogaster*, long-term imidacloprid treatment reduces brain dopamine levels, possibly via a blockade of nAChR-mediated dopamine release following persistent activation (Janner *et al*., 2021). This was partially true for honey bees, but only via additive interactions with QMP, as chronic exposure to 5 ppb imidacloprid exacerbated QMP's depression of brain dopamine levels. The depressive effects of QMP on dopamine have been discussed in previous chapters (Chapter three, Chapter four). Given the increased attraction to QMP associated with imidacloprid treatment, it is tempting to conclude that heightened interaction of imidacloprid-treated workers with QMP exacerbated QMP's depression of dopamine. In contrast with the original predictions of H2 (i.e. that imidacloprid directly modulates biogenic amines), this would instead imply that imidacloprid alters dopamine indirectly via this increased attraction to QMP. Workers that have not been exposed to QMP from adult emergence rapidly lose their ability to detect it, an effect mediated by QMP's effects on the gene expression of antennal odorant receptors (Vergoz *et al*., 2009). It would therefore be expected under this proposed model that workers housed without QMP see neither increased attraction to QMP nor interactive depression of brain dopamine levels following imidacloprid exposure, as was observed in this study.

Another factor that underlies worker attraction to QMP is worker reproductive capacity, where workers with smaller ovaries (i.e. fewer ovarioles) show greater attraction to the queen and her pheromones (Kocher *et al*., 2010). Ovariole numbers are determined during development (refer Section 1.2.1, General Introduction), and are under the control of genetic (Makert *et al*., 2006; Robinson *et al*., 1990) and environmental factors (Backx *et al*., 2012). For instance, stressors during development such as nutrition limitation reduce ovariole numbers in adult honey bees (Hoover *et al*., 2006). Though limited, there is also evidence of plasticity in ovariole numbers in adulthood via programmed cell death (Ronai *et al*., 2017), raising the possibility that imidacloprid exposure during adulthood could act as a stressor and reduce ovariole numbers. However, empirical support for this hypothesis is limited due to uncertainty over the extent to which plasticity in ovariole number is environmentally responsive in adulthood, and whether such responses could occur within the ten day time-frame of this experiment.

Although the mechanisms underlying imidacloprid's effects on retinue response behaviour require further study, the consequences of this increased attraction to the queen for whole-colony function can still be considered. QMP maintains temporal polyethism among workers, and increased QMP exposure (as simulated by supplemental QMP treatment of colonies) has been shown to delay the onset of foraging (Pankiw *et al*., 1998). Increased attraction, and hence exposure to, QMP could therefore decrease forager numbers in the hive by delaying the transition to foraging, leading to diminished food stores and poorer colony health. This idea is supported by a study of differentially expressed genes (DEGs) in honey bee workers following neonicotinoid exposure (Christen *et al*., 2016); In their study, neonicotinoid treatment led to an up-regulation of *vitellogenin,* a gene associated with delayed foraging onset. Through its suppression of JH titres in workers, vitellogenin is thought to act as regulator of behavioural maturation, with reductions in vitellogenin associated with the transition to foraging (Amdam & Omholt, 2002; Hartfelder *et al*., 2013). Indeed, down-regulation of *vitellogenin* using RNAi has been found to accelerate the behavioural shift to foraging and produce

precocious foragers (Flatt *et al*., 2013). As *vitellogenin* expression is regulated by QMP exposure (Nakaoka *et al*., 2008; Duncan, unpublished data - see Appendix L, Figure L.1), the increased attraction to QMP demonstrated here could provide a missing explanatory link between neonicotinoid treatment and up-regulation of *vitellogenin,* which has been shown to restrict the transition of workers to foraging. Evidence from the field has previously shown that neonicotinoid exposure reduces foraging activity in the hive, though this has formerly been attributed to disturbed memory formation (Fischer *et al*., 2014; Schneider *et al*., 2012). Here, imidacloprid's increased attraction of workers to QMP is proposed as an alternative or additional explanation for these previously reported forager losses.

In the present study, locomotor activity was measured as a proxy for foraging activity to test QMP's effects on the transition to foraging. There was no evidence to support an effect of QMP on locomotor activity levels in five-day old workers, nor for any disruptive effects of imidacloprid. However, the extent to which activity levels can truly be viewed as a proxy for foraging activity is limited. Firstly, although high levels of locomotor activity are predictors of good foraging performance (Klein *et al*., 2019), phototaxis (movement towards light) is more closely linked with the transition from work inside the hive to outside, such as foraging (Tosi & Nieh, 2017). Circadian differences in locomotor activity are also tied to the onset of foraging, as nurses carry out their tasks within the hive and therefore have no need to be responsive to daylight cycles (Bloch & Meshi, 2007). As the laboratory environment may intrinsically affect natural circadian rhythms, examining the phototaxis of workers would be a better behavioural proxy for testing the onset of foraging. Secondly, workers were tested at five-days old, which is likely too young for the transition to foraging to be observed, as this usually occurs between four and eight weeks into a worker's life (Ruepell *et al*., 2007). However, due to limitations of the lifespan of caged laboratory workers (refer Section 2.1.1, General Methods), studying laboratory workers over ten days old would not have been feasible and would require an entirely different experimental design. One possibility would be to use mark-release recapture to age-match workers (as used in Chapter three), releasing marked

workers into hives undergoing different imidacloprid exposure regimes and recapturing at different time points for behavioural testing. This would further test the hypothesis that neonicotinoid exposure delays the onset of foraging in workers with behavioural evidence, and retinue response assays could additionally be carried out to link the transition to foraging with responsiveness to QMP.

An unexpected but finding from this chapter is the speculative combined toxicity effect of imidacloprid with QMP. To my knowledge, previous laboratory-based assessments into the lethality of chronic neonicotinoid exposure have not been carried out in the presence of QMP (Reviewed by Cresswell, 2011). Based on these studies, the LC50 of imidacloprid is estimated to be 1,760 ppb, but findings presented here suggest this number may be lower when combined with QMP. In this study, QMP exposure reduced the survival rates of bees treated with just 5 ppb imidacloprid over ten days from 87% to 73% in experiment one.. At the low doses used here, these differences in mortality are relatively subtle, and this acute toxicity risk may not constitute much of a threat to hives as a whole. However, imidacloprid has been detected at concentrations of 6-206 ppb in the pollen stores of hives (Mullin *et al*., 2010), and possible combined toxicity effects with QMP at these upper levels are unknown. Until now, the concentrations of neonicotinoids encountered by bees have not been thought a concern for acute toxicity, with studies instead focusing on sub-lethal effects of these pesticides (e.g. Decourtye, Devillers, *et al*., 2004; Henry *et al*., 2012; Sandrock *et al*., 2014; Schneider *et al*., 2012; Williamson *et al*., 2014). If QMP enhances the lethality of imidacloprid, this raises renewed concern into the acute toxicity of this pesticide at field-realistic concentrations. Further assessment into the lethality of neonicotinoids, as well as novel pesticides undergoing development, in combination with QMP at a wider range of chronic doses is needed to address this potential underestimation of toxicity in laboratory studies.

As an economically important species providing essential agricultural pollination services, declines of honey bee populations are a worldwide concern. Laboratory studies are a useful tool for investigating pesticide toxicity, sublethal effects on behaviour and their underlying molecular mechanisms, but they lack many elements present in the

hive, such as social feedback loops and freedom of workers to exhibit 'natural' behaviours (Henry *et al*., 2015). It is demonstrated here that QMP is another important element that differs between laboratory and field-based studies. QMP is central to honey bee biology, and failure to include this pheromone in laboratory studies may cause consistent underestimation of neonicotinoid toxicity.

To my knowledge, the interactive effects of imidacloprid on key worker responses to QMP have also not previously been considered. Most notably, the increased attraction of workers to QMP caused by imidacloprid is predicted to have knock-on effects for hive functionality, and may be a driver of previously reported reductions of foraging activity in neonicotinoid-treated hives. Key future areas of study have been identified from this research, including 1. Improved understanding of the mechanism underlying imidacloprid's increase in worker attraction to QMP, specifically elucidating imidacloprid's effects on JH titres and antennal octopamine receptor expression, and 2. Empirical testing into whether imidacloprid's increased QMP attraction translates to delayed-onset foraging and an overall decline in hive foraging activity. Given the essential role of QMP in the maintenance of colony function, understanding the interactive effects of pesticides with this pheromone is an area which merits further research attention.

Chapter 7

General Discussion

7.1 Thesis Overview

The primary aim of this research was to improve our understanding of one of the major mechanisms maintaining colony cohesion in honey bees: the inhibition of worker reproduction by QMP (Hoover *et al*., 2003; Khila & Abouheif, 2008; Ratnieks *et al*., 2006). A substantial body of evidence already existed implicating biogenic amines, particularly dopamine, as mediators of QMP's command over the behaviour and ovarian physiology of workers (i.e. Beggs *et al*., 2007; Dombroski *et al*., 2003; Harris & Woodring, 1995; Sasaki & Nagao, 2001; Vergoz *et al*., 2012). This research aimed to bring further clarity to the roles played by biogenic amines by 1. focusing on the "missing link" between QMP's alterations to biogenic amine levels in the brain and how this comes to effect peripheral ovarian tissue (Chapter three), and 2. testing the causal relationship between biogenic amine titres (in particular dopamine) and ovary development through a series of manipulative feeding trials (Chapter four).

Given the accumulation of evidence that the depression of biogenic amines in the brain and hemolymph by QMP inhibits ovary development in workers, I then asked the question as to how queens manage to evade these effects of their own pheromone (Chapter five). I explored caste-specific differences in QMP exposure levels as a possible mechanism of self-sterilization evasion in queens, but emphasise that this is an emerging area of research which requires further study.

Finally, I aimed to apply my understanding of the molecular action of QMP to an applied issue: pesticide-driven declines of honey bee populations. In Chapter six I examined the interactive effects of QMP with a commonly used neonicotinoid pesticide, finding most notably a behavioural shift in imidacloprid-treated workers towards increased attraction to QMP. Given this pheromone's role in maintaining division of labour and colony harmony, this behavioural change may have implications for whole-colony function. These findings emphasize the significance of QMP to honey bee biology, and highlight a

need for the inclusion of this pheromone inclusion in future assessments of pesticide risks to honey bees.

7.2 What evidence is there that biogenic amines mediate QMP's control of worker reproduction?

7.2.1 Dopamine

Prior to this research, an accumulating number of studies proposed an involvement of dopamine in QMP's control of worker reproduction. Based on the finding that brain dopamine levels are reduced in QR workers (Harris & Woodring, 1995; Sasaki & Nagao, 2001), at least in part due to direct modulation by the QMP component HVA (Beggs *et al*., 2007), dopamine was identified as a key second messenger to QMP. This, along with the positive correlation between ovary development in QL workers (Sasaki & Nagao, 2001) and the acceleration of this development by dopamine feeding (Dombroski *et al*., 2003), led some to hypothesize that QMP inhibits worker reproduction by reducing dopamine titres in workers (i.e. Sasaki *et al*., 2021). While QMP's modulation of dopamine perhaps represented our best understood mechanism underlying QMP's effects on peripheral tissues such as the ovary, the evidence thus far was still largely circumstantial.

In this thesis, additional evidence was found to support an involvement of dopamine in mediating QMP's inhibition of reproduction. In Chapter three, the reduced brain dopamine in workers from QR relative to QL hives was consistent with previous work (Beggs *et al*., 2007; Harris & Woodring, 1995; Sasaki & Nagao, 2001). Further, the finding that brain dopamine levels correlated positively with ovary activity in workers from QL laboratory microcolonies (Chapter three) confirmed the results of past studies (Sasaki & Nagao, 2001). The depression of dopamine was causally linked with ovarian repression in Chapter four, in which treatment with the dopamine synthesis inhibitor 3-iodo-L-tyrosine (iodotyrosine) blocked ovarian development in workers to a degree comparable with QMP exposure. This finding would be strengthened by an assessment of internal dopamine levels in iodotyrosine-fed workers to confirm A. its efficacy as a depressor of

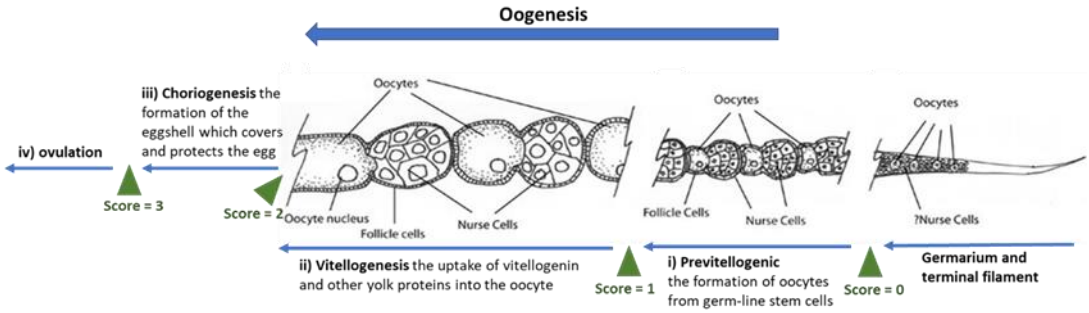

Figure 7.1 Stages of oogenesis in a honey bee ovary. Diagram the morphology of an active honey bee ovariole showing the distinct stages of oogenesis: i) previtellogenic, ii) vitellogenesis, iii) choriogenesis and iv) ovulation. The production of mature oocytes can be thought of as occurring on a 'conveyor belt' of development going from right to left. However, reaching complete oocyte maturity is not an inevitable process, and ovary development in workers frequently does not progress beyond certain stages of oogenesis. The corresponding ovary score associated with the completion of each stage is indicated by green arrowheads. For images of each ovary score, see also Figure 2.2.

dopamine levels and B. whether its effects were tissue specific, i.e. to the brain or the hemolymph. However, considered alongside evidence for a role of dopamine in regulating reproduction in other species (e.g. *Polistes,* Sasaki *et al*., 2007; fire ants, Boulay *et al*., 2001; bumble bees, Bloch *et al* 2000, Sasaki *et al*., 2017; refer Section 1.5.3, General Introduction), the findings from Chapters three and four add to an accumulating body of evidence that QMP's depression of dopamine contributes to its sterilizing effects in workers.

Preliminary evidence from this thesis indicates that dopamine's role could be to mediate the earliest stages of ovarian development. In Chapter thee, brain dopamine levels were found to correlate with ovary activity during the initial stages of oogenesis (i.e. scores 0-2; Figure 7.1). However, the drop in brain dopamine levels between the ovary stages of two and three (refer Figure 3.6) indicates a possible stabilising of dopamine synthesis once full ovarian maturity is reached. The ovary development of QL workers was found to be accelerated in laboratory microcolonies relative to the hive (Chapter three), possibly due to their improved nutrition from their diet of CBF (refer section 2.1.1, General Methods). Consistently throughout this thesis (Chapters three, five and six), there has been no difference in brain dopamine levels between 10-day old workers from

QR and QL laboratory microcolonies, despite clear differences in ovary activity. One possible interpretation of this is that as a number of laboratory-maintained workers already possess mature oocytes by 10-days old, this timepoint is too late to observe differences in dopamine levels that precede this point of ovary maturity. In the hive, where ovary development is slower, brain dopamine levels are clearly elevated in QL relative to QR workers (Chapter three; Harris & Woodring, 1995). In laboratory studies, however, differences in brain dopamine levels between QL and QR workers have only ever been observed at 2-days old (Beggs *et al*., 2007). At 2-days old, laboratory workers are still at the beginning of the pre-vitellogenic stage (i.e. developing from a score of 0 into a 1; Figure 7.1; Koudjil & Doumandji, 2008), whereas by 10-days old many workers are reaching peak vitellogenic stage and mature oocytes are being formed (i.e. developing from a score of 2 into a 3; Figure 7.1; Koudjil & Doumandji, 2008). Therefore, the initial elevation of dopamine following the loss of the queen may be the trigger promoting the onset of early stages of ovary development such as previtellogenesis and vitellogenesis (Figure 7.1). Given that Notch signalling also regulates development in the ovary at the earliest stages of oogenesis (Duncan *et al*., 2016), this is consistent with the idea that dopamine levels effect ovary activity by modulating the activity of Notch signalling in the ovary. This idea is discussed in more depth in section 7.3.3.

Future studies should seek to empirically test the hypothesis that dopamine regulates the earliest stages of oogenesis. A key experiment to test this would be to examine the temporal dynamics of changes in dopamine levels in QL laboratory workers at a series of timepoints so that changes preceding the onset of full ovarian maturity by day 10 can be captured. As the processes by which dopamine levels are modulated by the presence of QMP are not fully understood (though the QMP component HVA is thought to be involved; Beggs *et al*., 2007), it would also be of interest to measure the expression of genes involved in dopamine biosynthesis using RT-qPCR. The processes by which QMP may regulate dopamine levels in workers are further discussed in Section 7.3.2.

7.2.2 Octopamine

In addition to dopamine, I also found evidence for a role of octopamine in mediating QMP's effects. In Chapter three, I demonstrated that worker octopamine titres in both the brain and hemolymph are depressed by QMP. Octopamine is known to act alongside JH as a behavioural pacemaker promoting the onset of foraging (Wagener-Hulme *et al*., 1999), but responsiveness of this amine to QMP has not previously been demonstrated.

I speculate that there may be a role of octopamine alongside dopamine as a mediator of QMP's inhibition of worker reproduction. In Chapter three, I observed a positive correlation between brain octopamine titres and ovary activity in QL workers (Figure 3.6), though this was not statistically significant so should be interpreted with caution. However, the fact that the octopamine receptors *AmOA1* and *OAβ2R* are expressed in the honey bee ovary alongside dopamine receptors (Vergoz *et al*., 2012; Duncan, unpublished data) also supports a role of octopamine in mediating ovary development. Octopamine is involved in the regulation of reproduction in a range of insects; For instance, egg laying is stimulated by the injection of octopamine in the rice leaf bug (Yamane, 2013) and the diamondback moth (Li *et al*., 2020). In *D. melanogaster,* octopamine regulates ovulation and the muscle contractions involved in egg-laying (Meiselman *et al*., 2018; White *et al*., 2021). It is therefore likely that a conserved gonadotropic function of octopamine might also exist in honey bees.

As was carried out in Chapter four for dopamine, experimental manipulations of octopamine levels are necessary to test whether octopamine is a positive regulator of reproduction in honey bees. One study has addressed this (Salomon *et al*., 2012), finding that oral octopamine treatment did not increase the propensity of QL workers to activate their ovaries. However, octopamine's role in *D. melanogaster* is in the regulation of oviposition (Meiselman *et al*., 2018; White *et al*., 2021), and if this function is conserved in honey bees one may not expect to see an effect on ovary development. In addition, octopamine treatment did not cause an elevation of octopamine titres in the brain in Salomon *et al*., (2012)'s study, leading to uncertainty as to the effectiveness of

dietary octopamine supplementation as a delivery method. As was discussed in Chapter four with respect to dopamine supplementation methods, more sophisticated methodology than dietary administration of biogenic amines is required to better understand their role in regulating reproduction. In *D. melanogaster*, the GAL4/UAS system has been used to study octopamine's role in reproduction by manipulating the expression of octopamine synthesis and receptor genes (Deady & Sun, 2015; Lee *et al*., 2009; Li *et al*., 2015; Lim *et al*., 2014). While the genetic tools available to honey bees are less advanced, knockdown of octopamine synthesis and receptor genes using RNAi (i.e. Jarosch & Moritz, 2011; Maori *et al*., 2019), or oral application of an octopamine synthesis inhibitor could instead be used to investigate the potential role of octopamine in honey bee reproduction.

7.3 Mechanism of QMP-biogenic amine signalling

As well as testing the hypothesis that biogenic amines mediate QMP's induction of sterility, this research aimed to shed light on the specific mechanisms involved in this signalling pathway. The action of QMP can be broken down into three stages as depicted in Figure 7.2. 1.) Detection - this is the process by which QMP is initially sensed, 2.) Transmission - the process by which QMP's signal is transmitted to the ovaries, and 3.) Ovarian response - the processes acting in the ovary itself controlling development. In the above section, I presented the evidence we have for roles of dopamine and octopamine as mediators in this process. In the following section, I discuss what is known about the possible mechanisms of biogenic amine-QMP signalling with consideration of each of the three stages identified above.

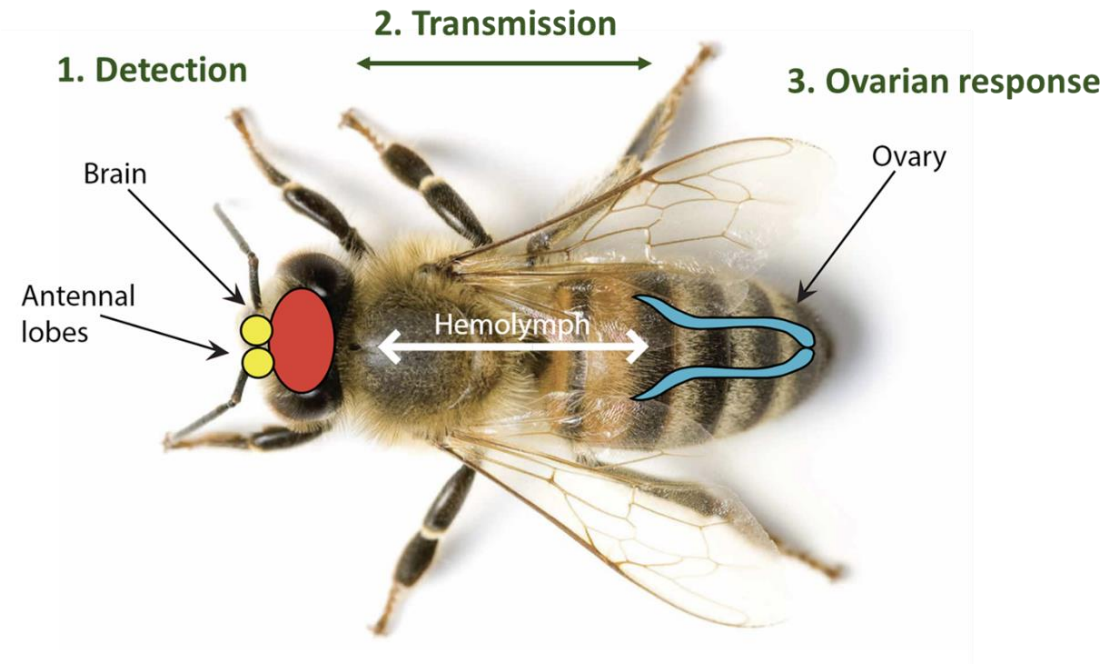

Figure 7.2 Model of the transmission of QMP's signal to the ovary consisting of three stages: **1. Detection:** the process by which QMP is initially sensed, **2.) Transmission:** the process by which QMP's signal is transmitted to the ovaries, and **3.) Ovarian response**; the processes acting in the ovary itself controlling development.

7.3.1 How is QMP detected?

Investigating the modes of QMP detection by workers (Figure 7.1; Stage 1) did not fall within the scope of this thesis. However, this research has emphasised the gaps in our knowledge regarding this stage of the QMP signalling process.

Current understanding of QMP transfer throughout the hive is that QMP is picked up by nurses attending the queen (Allen, 1955) and distributed through the colony via worker-worker interactions such as antennation, grooming and trophallaxis (Naumann *et al*., 1991). This indicates that there are two possible modes of QMP uptake by workers: antennal (i.e. through worker-worker antennation) and oral (i.e. through grooming and trophallaxis). Physical contact with QMP has been shown to be necessary to induce full inhibition of ovary activity (Katzav-Gozansky *et al*., 2004; Lovegrove *et al*., 2020), confirming that QMP components are non-volatile. However, the relative importance of antennal vs oral detection methods cannot be deduced from this.

The mode of QMP uptake is relevant for our understanding of the downstream processes involved in regulating biogenic amines (Chapter three and Chapter four). Antennal detection implicates direct neuronal connections with the brain as being involved in the transmission of QMP's signal (Carcaud *et al*., 2015). Previous work has shown that QMP induces neuronal responses in the antennal lobe of honey bees (Roussel *et al*., 2014), though this was linked only to behavioural responses to QMP and effects on ovarian development were not measured. Whether neuronal pathways are also involved in mediating QMP's effects on reproduction therefore remains unclear. Oral uptake, on the other hand, would likely bypass neuronal signalling channels and instead exert effects via the circulatory system. It is unclear whether antennal or oral exposure to QMP act alone or in combination to induce the effects of QMP. Future studies should focus on developing an assay that enables workers to physically antennate, but not ingest, QMP to determine whether the full suite of behavioural and physiological responses to QMP can be induced through antennal exposure alone.

Understanding the relative importance of these QMP uptake methods would also be a valuable addition to the work addressed in Chapter five, which focused on understanding the mechanisms behind QMP's lack of repressive effects in queens. In addition to the dose-specific effects of QMP found in chapter five, differential QMP uptake between queens and workers could hypothetically also be a driver of self-sterility evasion. For instance, seeing as the most likely route of QMP re-internalisation by queens is through ingestion (Naumann *et al*., 1991; although this remains to be definitively tested), the lack of antennal QMP exposure in queens could mean that possible neuronal effects of QMP on ovary activity are bypassed. Further study assessing the relative importance of antennal vs oral QMP uptake is thus essential not only for appreciating the downstream processes mediating QMP's transmission, but also for the question of queen self-sterility evasion.

7.3.2 How are dopamine and octopamine modulated by QMP and transmitted to the ovary?

Research into the molecular action of QMP thus far has been centered predominantly on QMP's effects on the brain (i.e. Beggs *et al*., 2007; Beggs & Mercer, 2009; Carcaud *et al*., 2015), with little work addressing how these changes are linked with the modulation of peripheral ovary tissue. A key finding from this research was the detection of dopamine and octopamine in the hemolymph (Chapter three). This provides evidence that the activity of these amines on ovary development may be mediated at least in part by their functions as circulating neurohormones. Hemolymph levels of dopamine and octopamine were also both reduced in workers from QR relative to QL hives (although reductions in hemolymph dopamine were not statistically significant; Chapter three), suggesting either direct or indirect modulation by QMP or other queen pheromones.

For dopamine, its presence in the hemolymph begins to bridge the gap in our understanding of the transmission of QMP's signal to the ovary (Stage 2; Figure 7.2). However, the link between brain and hemolymph dopamine levels is unclear. In male honey bees, dopamine increases with age in the brain and hemolymph in parallel to mediate the onset of mating behaviour (Sasaki & Watanabe, 2022), with hemolymph dopamine thought to interact with the dopamine receptors AmDop1, Amdop2 and AmDop3 in the testes (Matsushima *et al*., 2018). However, it is not known whether the same processes regulate dopamine levels in the brain and hemolymph, or if these represent independent mechanisms. The source of biogenic amines in the hemolymph is unclear; For instance, they could be synthesized in the hemocytes (i.e. as in the moth *Chilo suppressalis;* Wu *et al*., 2015), secreted from peripheral tissues such as the fat body, or even secreted from the brain itself.

QMP is known to regulate dopamine levels in the brain at least in part by HVA, a component structurally very similar to dopamine which has the potential to bind to the dopamine receptor AmDop3 as an agonist (Beggs & Mercer, 2009). However, the mechanism behind HVA's effects on brain dopamine has not been fully elucidated. Sasaki

& Watanabe (2022) proposed two ways in which HVA may control brain dopamine: 1. Through antennal detection of HVA and the transmission of neural signals to influence brain dopamine levels, and 2. Through oral HVA intake, which modulates brain dopamine via the hemolymph. While the neural responses to HVA in the antennal lobes support the former (Carcaud *et al*., 2015), the discovery of potentially QMP-responsive dopamine in the hemolymph supports the latter (Chapter three). It is possible that antennal and oral QMP detection modulate dopamine via different pathways simultaneously to one another; For instance, while antennal detection modulates neural signals in the brain (Carcaud *et al*., 2015), oral QMP uptake may bypass the brain to directly or indirectly effect hemolymph dopamine. As discussed above (Section 7.3.1), studying the relative importance of antennal vs oral mechanisms of QMP uptake would aid our understanding of QMP's modulation of dopamine in each uptake pathway.

Less is known about the process by which QMP may modulate octopamine levels. Beggs & Mercer (2009) show that HVA's activation of the dopamine receptor AmDop3 in the brain is highly selective, with neither agonist nor antagonist activity on the octopamine receptor AmOA1. However, the role of receptor activation in HVA's modulation of dopaminergic pathways is unclear, and the effect of HVA on octopamine levels has not been definitively tested. The involvement of other pheromone components in the modulation of octopamine is also uncertain, and a screening of the effects of each component on octopamine titres would be a useful way to identify components worthy of further investigation. Such a screen should include, but not be limited to, the five major QMP components: 9-ODA, both enantiomers of 9-HDA, HOB and HVA (Slessor *et al*., 1988). However, it is important to note that QMP alone does not inhibit worker reproduction as effectively as a real queen (Maisonnasse *et al*., 2010). Given the finding that octopamine was lowered in the hemolymph only in workers exposed to a real queen (as opposed to synthetic QMP alone; Chapter three), this highlights the potential importance of additional pheromones to QMP in the modulation of octopamine. Other queen components to examine include tergal gland compounds, which have also been implicated in mediating ovarian development (Princen *et al*., 2019; Wossler & Crewe,

1999), and brood pheromone (Maisonnasse, Lenoir, *et al*., 2010). In addition to octopamine, it would also be of value to assess the effects of these compounds on dopamine levels, as it has not yet been determined whether other compounds act in addition to HVA to modulate dopamine levels. Similarly to octopamine, dopamine levels were also reduced to a lesser extent by synthetic QMP than by a real in-hive queen (Chapter three), and a second possible interpretation of this (i.e. in addition to the temporal interpretation discussed in section 7.2.1) is the existence of additional modulatory compounds beyond the five major QMP components that are included in the synthetic blend.

When it comes to attempting to understand how octopamine may regulate reproduction in honey bees, *D. Melanogaster* is our best understood model. In *D. melanogaster,* Octopamine's effects on reproduction are mediated in part by the activity of octopaminergic neurons, which innervate peripheral tissues including the reproductive tract (Pauls *et al*., 2018) as well as being located throughout the brain (Busch *et al*., 2009; Sherer *et al*., 2020). There is also evidence that octopamine effects reproduction indirectly via regulation of the gonadotropins 20E and JH (Gruntenko *et al*., 2007; Rauschenbach *et al*., 2007). In honey bees, given the loss of gonadotropic function of JH (Rodrigues & Flatt, 2016), regulation of reproduction by octopamine would perhaps be more likely to function through direct interaction with ovarian tissue, either via neuronal connectivity, circulatory octopamine in the hemolymph or a combination of both. Support from the latter comes from the finding in Chapter three that octopamine was present in the hemolymph, and repressed by QMP in parallel with the brain. The relative importance of neuronal vs circulatory systems in the transduction of octopamine to the ovary may depend on the mode of detection used by workers to sense QMP, with antennal detection perhaps being more directly wired with neuronal connections than oral uptake.

In this research, I have demonstrated the potential for biogenic amines to act on the ovary via the circulatory system (Chapter three). However, the activity of octopaminergic and dopaminergic neurons was not assessed. It is possible that these two pathways act

together in the transduction of QMP's signal to the ovary, perhaps reflecting a degree of functional redundancy over antennal and oral QMP exposure. Further study using electrophysiology to examine the neuronal control of ovary development in honey bees would be a valuable addition to our knowledge, as have been carried out in *D. melanogaster* (Clark & Lange, 2003; Rodríguez-Valentín *et al*., 2006) and the locust *Locusta migratoria* (Wong & Lange, 2014).

7.3.3 How is the ovarian response coordinated?

The final aspect of the mediation of QMP's effects on ovary development is the ovarian response (Figure 7.2; Stage three). Dopamine and octopamine could hypothetically mediate ovary physiology directly through activity with the ovarian receptors AmDop1, AmDop3 and AmOA1 (Vergoz *et al*., 2012). QMP also effects the expression of these receptors (Vergoz *et al*., 2012), and these changes in expression may act in parallel with modulation of dopamine and octopamine titres in the hemolymph to control ovary development, as is discussed in Chapter three.

There is preliminary evidence suggesting a link between dopamine receptor activation and Notch signalling in the ovary. Notch signalling acts in the germarium, the region of the ovary where germ cells are specified (see Figure 7.1), to actively inhibit oogenesis in response to the presence of QMP (Duncan *et al*., 2016). Expression of the dopamine receptor *AmDop1* is also localised to the germarium (Duncan, unpublished data; see Appendix F, Figure F.1), and is up-regulated in two day old QL workers (Vergoz *et al*., 2012). Assuming AmDop1 receptor activity is involved in regulating ovary development via the Notch signalling pathway, this is consistent with the idea that dopamine's role is to regulate the earliest checkpoints in oogenesis (as discussed in section 7.2.1). However, further study is required to ascertain whether there are links between ovarian biogenic amine receptor activity and the regulation of Notch signalling. This could theoretically be tested using RNAi to knockdown receptor expression in the ovaries and measuring the effects on ovary activation and the expression of Notch-responsive genes *Her* and *bHLH2* using RT-qPCR However, such an experiment is dependent on successful

tissue-specific gene knockdown, which we do not currently have the tools to achieve. In the absence of tissue-specific RNAi, the knockdown of biogenic amine receptors in the whole body would likely disrupt many fundamental processes and as such have lethal effects. An alternative approach would be to treat QR worker ovaries ex-vivo (i.e. in culture) with the levels of dopamine and octopamine found to be circulating in Chapter three. This could be carried out in the presence and absence of a transcriptional inhibitor, and RNA-seq could be used to assess the primary transcriptional response of the ovary to dopamine and octopamine. Such a study would confirm whether dopamine and octopamine's direct interactions with receptors in the ovary regulate Notch-mediated reproductive constraint.

7.4 Worker reproduction is likely constrained by multiple redundant processes

While this thesis focused on understanding the role of just one candidate pathway that may be targeted by QMP, it also shines a light on the fact that biogenic amines are unlikely the sole mediators of QMP's effects. This is most apparent from findings in Chapter four, in which I observed that supplementary dopamine feeding did not restore ovary activity in workers exposed to QMP. In light of the hypothesised role of octopamine in regulating reproduction alongside dopamine (Chapter three), it is possible that co-administration of both dopamine and octopamine would be required to overcome QMP's inhibitory effects. An experiment to test this would be simple to conduct and would add to existing evidence for octopamine's involvement in mediating ovary repression.

This being said, there was a repeated occurrence throughout the thesis of inhibited ovary development in the absence of reductions to dopamine and octopamine in workers maintained in the laboratory (Chapter three, chapter five, chapter six). In experiments conducted using laboratory microcolonies, brain octopamine was inconsistently lowered by synthetic QMP (Chapter three, Chapter five, Chapter six), and brain dopamine was lowered by synthetic QMP only in conjunction with imidacloprid

treatment (Chapter six). Neither dopamine nor octopamine were ever significantly lowered in the hemolymph by synthetic QMP in laboratory studies (Chapter three, Chapter six). Despite this, ovary activity was consistently inhibited by synthetic QMP across all chapters in the thesis. One interpretation of this, that it is the result of the temporal dynamics of ovary development in the lab vs the hive, was discussed above (Section 7.2.1), and requires further study over a range of timepoints to explicitly test. However, an alternative explanation is that synthetic QMP was inhibiting worker reproduction via a pathway that did not involve biogenic amines. This is at odds with the fact that HVA, a QMP component present in the synthetic blend used, has been directly implicated in the modulation of dopamine (Beggs *et al*., 2007). However, Beggs *et al*., (2007) exposed workers to a much greater amount of HVA than would typically be received within the QMP blend, in which 1 queen equivalent (QE) consists of just 2µg HVA (Slessor *et al*., 1988) which is disseminated among tens of thousands of workers in the hive (Naumann *et al*., 1991).

The idea that synthetic QMP may have inhibited reproduction through a process additional to biogenic amines is consistent with the finding that honey bee queens possess a functionally redundant set of pheromones (Princen *et al*., 2019). Importantly, Princen *et al*., (2019) demonstrate that worker ovary activity is repressed by the QMP components 9-ODA and 9-HDA, as well as tergal gland esters, as effectively as the full QMP blend. With the exception of HVA (Beggs *et al*., 2007; Beggs & Mercer, 2009), little is known about the molecular action of other QMP components and queen pheromones, and this represents a crucial gap in our knowledge. While the modulation of biogenic amines represents our best-studied mechanism behind QMP's effects on worker ovaries, research into alternative pathways of mediation by the full range of the queen's pheromone components should not be neglected. In *D. melanogaster*, for instance, there is evidence that QMP inhibits oogenesis by modulating insulin signalling, causing an arrest in ovary development not dissimilar to a starvation response (Lovegrove *et al*., 2021). A more holistic view of how QMP and other queen pheromones modulate the

broader neuroendocrine system is therefore essential to our understanding of how the queen maintains sterility in honey bee workers.

7.5 Queen control vs queen signal hypotheses

Whether queen pheromones more generally function as honest signals for the presence of a fecund queen (Keller & Nonacs., 1993; Monnin, 2006; Seeley, 1985) or as manipulative compounds that chemically enforce sterilization upon workers against their reproductive interests (Fletcher & Ross, 1985; Strauss *et al*., 2008; Hefetz, 2004; Hölldobler & Wilson, 1990) has been a source of much debate. These two contrasting hypotheses as to how queen pheromones may have evolved are known as the honest signal and queen control hypotheses (refer Section 1.4, General Introduction). Relative to the conserved class of cuticular hydrocarbon-based pheromones that constitute the queen pheromones of most eusocial hymenopterans (van Oystaeyen *et al*., 2014), QMP is highly derived and structurally complex (Hoover *et al*., 2003; Slessor *et al*., 1988; Refer Section 1.3.2, General Introduction). This contributes towards speculation that unlike cuticular hydrocarbons which likely arose from ancestral fertility signals (i.e. in line with the queen signal hypothesis; Chapuisat, 2014), QMP could have taken on a novel function as a chemical suppressor of reproduction (i.e. in line with the queen control hypothesis). Indeed, QMP's ability to repress reproduction across a broad phylogeny of species suggests that this highly derived queen pheromone may be targeting ancestral pathways involved in regulating reproduction (e.g. *D. melanogaster:* Camiletti *et al*., 2013; ant species: Carlisle & Butler., 1956; house fly: Nayar, 1963; termite: Hrdy *et al* 1960), suggesting a role as a chemical suppressor of reproduction. Dopamine and octopamine are thought to have roles in reproduction in a range of insect species (e.g. *D. melanogaster:* Lim *et al*., 2014, Rodríguez-Valentín *et al*., 2006; *Polistes*: Sasaki *et al*., 2007; fire ants: Boulay *et al*., 2001; bumble bees: Bloch *et al* 2000). Therefore, the modulation of these biogenic amines by QMP or other queen pheromones could be said to support the queen control hypothesis, particularly as it has been demonstrated that dopamine in particular is required for ovary development (Chapter four - inhibiting

dopamine biosynthesis reduced ovary development in QL workers). Further, the fact that cuticular hydrocarbons do not share the same broad phylogenetic repressive effects as QMP (Lovegrove *et al*., 2019) is consistent with the idea that QMP's possible role as a chemical suppressor of reproduction is a novel feature not seen in other more chemically and functionally simple queen pheromones.

This thesis also highlighted that QMP likely uses multiple processes to constrain reproduction, with biogenic amines representing just one aspect of a complex picture (discussed in Section 7.4). The finding that honey bee queens possess a functionally redundant set of pheromones has previously been argued in support of the queen signal hypothesis (Princen *et al*., 2019). Princen *et al*., (2019) argue that the complex combination of queen pheromone compounds could increase the information content of the queen's signal, making it a more reliable indicator of fecundity. However, the fact that QMP contains multiple bioactive compounds could equally be argued to support the queen control hypothesis. For instance, under the queen control model, it is predicted that queen pheromones should evolve rapidly owing to an evolutionary 'arms race' between queens a workers, whereby workers are continually selected to evade repression and queens are under pressure to evolve novel repressive agents (Heinze & D'Ettorre, 2009). The functional redundancy of QMP, along with additional honey bee queen pheromones, would be an expected outcome of such a queen-worker arms race.

A crucial contradiction of the queen control hypothesis is how the queen could evade any chemically repressive effects of her own pheromone. In Chapter five, this question was addressed, and the demonstrated dose-specific effects of QMP represent a possible mechanism underlying caste-specific differences in QMP's inhibition of reproduction. However, further study quantifying the specific dosages of QMP received by queens and workers is integral to the development of this theory. Further, as discussed previously (Section 7.3.1), the relative importance of antennal and oral QMP uptake deserves further study, as different routes of QMP exposure in queens and workers is also a possible mechanism by which caste-specific repressive effects of QMP could arise. While the findings in Chapter five do not completely negate the contradiction of queen

fecundity inherent to the queen control hypothesis, they provide a useful framework from which future study can be directed.

7.6 Applied Research

Given the apparent complexity of QMP's effects on the neuroendocrine system in workers, in Chapter six I sought to determine whether this balance is disrupted by an environmental stressor: pesticide exposure. I tested the effects of a commonly used neonicotinoid pesticide, imidacloprid, on established worker responses to QMP, ranging from molecular, to physiological, to behavioural responses.

A key finding from this chapter was the increased attraction of imidacloprid-treated workers to QMP. Though the underlying mechanism behind this behavioural shift requires further study, its consequences have the potential to disrupt whole-colony function. Supplemental QMP exposure has been found to delay the behavioural shift of workers from nurses to foragers in the hive (Pankiw *et al*., 1998). Therefore, by increasing the attraction of workers to QMP, imidacloprid may lead to reduced forager recruitment in the hive, causing diminishing food stores and reduced colony productivity. This hypothesis is supported by empirical evidence from the field, in which neonicotinoid exposure has been found to reduce foraging activity in the hive (Fischer *et al*., 2014; Schneider *et al*., 2012). However, further study is required to robustly test this hypothesis. Firstly, an improved understanding of the mechanism underlying imidacloprid's increase in worker attraction to QMP is needed, with possible target pathways involving the modulation of JH titres and antennal expression of the octopamine receptor *AmOA1* which is thought to correspond with worker attraction to QMP (Mcquillan *et al*., 2014; Vergoz *et al*., 2009). Secondly, empirical testing into whether an increase in attraction to QMP translates to delayed-onset foraging and an overall decline in foraging activity of the hive is required.

A second speculative finding from chapter six was the apparent increase in imidacloprid lethality when administered in combination with QMP. Though not consistent between experiments, QMP exposure was seen to reduce the survival rates of workers from

laboratory microcolonies chronically exposed with 5 ppb imidacloprid from 87% in the absence of QMP to 73% in the presence of QMP. While these differences in mortality may be relatively subtle, it is worth noting that imidacloprid has been detected at concentrations of 6-206 ppb in the pollen stores of honey bee hives (Mullin *et al*., 2010), and the combined toxicity effects with QMP at the upper end of this range is unknown. To my knowledge, previous laboratory-based assessments into the lethality of chronic neonicotinoid exposure have not been carried out in the presence of QMP (Reviewed by Cresswell, 2011), and interactivity with QMP is not a standard test that is considered during the screening process for the approval of the use of pesticides. This makes a case for the inclusion of QMP in future studies into the lethality of existing pesticides to honey bees, and equally of novel pesticides during their development.

Why such a combined toxicity effect of imidacloprid and QMP may exist is not fully understood. Based on other findings from this thesis, it is possible that the exacerbated depression of brain dopamine levels in the presence of both QMP and imidacloprid could contribute towards increased mortality. In chapter four, treating workers with the dopamine synthesis inhibitor iodotyrosine at a concentration of 0.5 mg/mL led to a marked increase in mortality, indicating that a certain amount of dopamine is required for basic survival functions. Long-term imidacloprid exposure causes a reduction in brain dopamine levels in *D. melanogaster* (Janner *et al*., 2021), and the depression of dopamine by QMP and other queen pheromones has been demonstrated in this thesis (Chapter three). It is possible that this combination of processes, exacerbated by the increased attraction of workers to QMP (and hence increased exposure to its depressive effects), may reduce dopamine levels in workers to a point that survival is compromised.

7.7 Concluding remarks

A number of theories propose the ultimate reasons as to *why* eusociality may evolve, and these form an essential framework for reconciling the evolution of a sterile worker caste with the theory of evolution by natural selection (i.e. Hamilton, 1964b, 1964a; Nowak *et al*., 2010; Wilson & Wilson, 2007). However, it is an appreciation of the proximate mechanisms by which worker sterility is maintained that informs us *how* the social structures as advanced as those seen in the eusocial insects came to be.

By negating conflict over queen-worker reproduction, QMP is central to the maintenance of colony harmony in honey bees (Hoover *et al*., 2003; Khila & Abouheif, 2008; Ratnieks *et al*., 2006). This thesis has provided an in-depth exploration into just one possible neuroendocrine signalling pathway by which QMP may exert its inhibition of ovary development in workers, finding that octopamine and dopamine are likely involved in the mediation of reproductive constraint. However, it also shines a light on the fact that through its multiple levels of redundancy (i.e. Princen *et al*., 2019), QMP may modulate the reproduction of workers via a plethora of different possible signalling pathways. Not only this, but it would appear that QMP is unlikely alone in its regulation of reproduction, with additional pheromones such as tergal esters and brood pheromone also implicated in the repression of ovary activity (Princen *et al*., 2019; Wossler & Crewe, 1999; Maisonnasse, Lenoir *et al*., 2010). The level of complexity within this system thus cannot be overstated. How such a functionally complex network of pheromones came to be, consisting of both suppressive agents alongside honest indicators of fertility, has the potential to teach us many lessons in how eusociality has arisen and is maintained. Further study into the specific mechanisms governing reproductive constraints is therefore essential to the progression of our understanding of the fascinating complexity of eusocial insect societies.

Appendix A

HPLC-FLD standard calibration curves

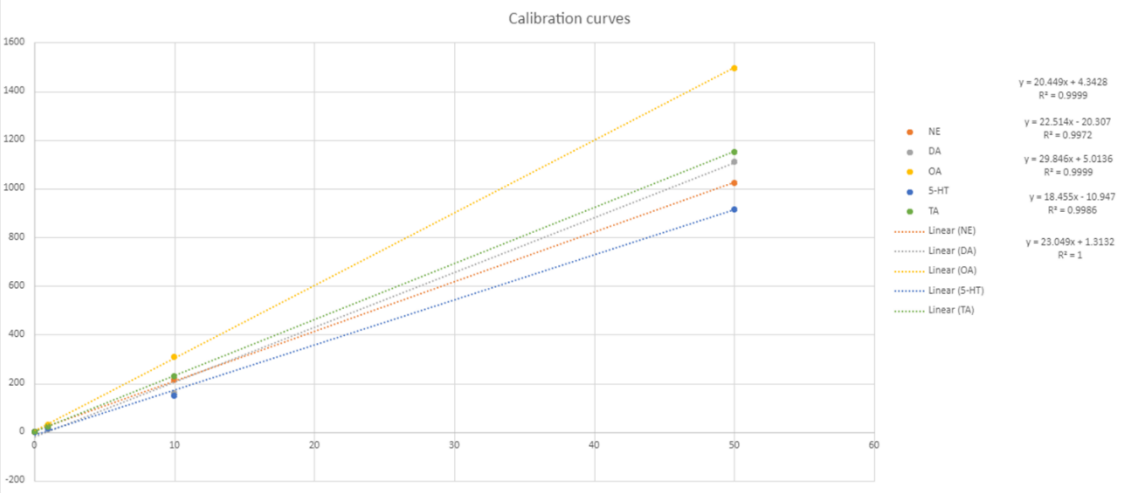

Figure A.1 HPLC-FLD standard calibration curves An example of a calibration curve used to quantify biogenic amines in biological samples from standards of known concentration.

Appendix B

Table of *post-hoc* pairwise comparisons from Chapter 3

Brain Dopamine					
Contrast	Estimate	SE	df	Z ratio	P value
QL Day 10 – QL Day 21	0.000486	0.000546	Inf	0.890	0.8100
QL Day 10 – QR Day 10	-0.001287	0.000454	Inf	-2.836	0.0236*
QL Day 10 – QR Day 21	-0.002214	0.001080	Inf	-2.051	0.1694
QL Day 21 – QR Day 10	-0.001773	0.000626	Inf	-2.833	0.0239*
QL Day 21 – QR Day 21	-0.002700	0.000985	Inf	-2.740	0.0313*
QR Day 10 – QR Day 21	-0.000927	0.001122	Inf	-0.826	0.8419
Brain Octopamine					
Contrast	Estimate	SE	df	Z ratio	P value
QL Day 10 – QL Day 21	-0.000477	0.000286	Inf	-1.652	0.3492
QL Day 10 – QR Day 10	-0.001135	0.000347	Inf	-3.258	0.0062**
QL Day 10 – QR Day 21	-0.003423	0.001342	Inf	-2.549	0.0528
QL Day 21 – QR Day 10	-0.000658	0.000412	Inf	-1.599	0.3791
QL Day 21 – QR Day 21	-0.002946	0.001342	Inf	-2.195	0.1246
QR Day 10 – QR Day 21	-0.002288	0.001374	Inf	-1.665	0.3425
Hemolymph Octopamine					
Contrast	Estimate	SE	df	Z ratio	P value
QL Day 10 – QL Day 21	-4.10e-04	0.000120	Inf	-3.403	0.0037**
QL Day 10 – QR Day 10	-4.24e-04	0.000133	Inf	-3.180	0.0080**
QL Day 10 – QR Day 21	-1.63e-03	0.000426	Inf	-3.828	0.0007***
QL Day 21 – QR Day 10	-1.43e-05	0.000163	Inf	-0.088	0.9998
QL Day 21 – QR Day 21	-1.22e-03	0.000434	Inf	-2.810	0.0255*
QR Day 10 – QR Day 21	-1.21e-03	0.000438	Inf	-2.750	0.0303*

Table B.1 Pairwise comparisons of biogenic amine levels in QR/QL workers on days 10/21
Post-hoc comparisons of dopamine levels in the brain and octopamine levels in the brain and hemolymph between all pairwise combinations of QL and QR workers at days 10 and 21. *Post-hoc* testing was computed using estimated marginal means on the maximal GLMM. GLMMs were fitted with inverse link functions, hence contrast estimates are still on the inverse scale. P-values are Tukey adjusted.

Appendix C

Assessment of dopamine dosages received between treatments

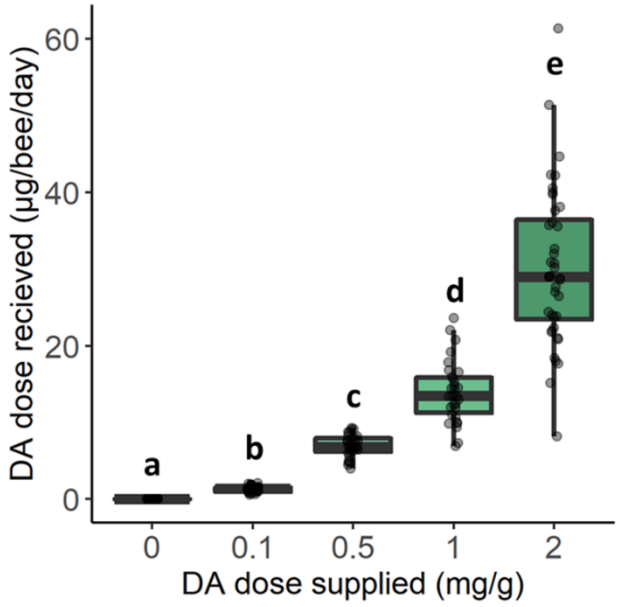

Figure C.1 Actual dopamine doses received per bee per day. The actual dopamine doses received in each treatment group were calculated by multiplying the mean food intake (mg/bee/day) for each treatment by the concentration of dopamine supplied in the food. The actual quantity of dopamine received differed significantly between treatments (Kruskall-Wallis; $\chi^2 = 150.4$, df = 4, $p < 0.001$). Pairwise comparisons were made using Wilcoxon rank sum tests with correction for multiple testing (see Table C.1). Statistically significant pairwise comparisons are denoted by boxplots not sharing a letter.

	0	**0.1**	**0.5**	**1**
0.1	1.3e-12	-	-	-
0.5	1.3e-12	<2e-16	-	-
1	1.3e-12	<2e-16	1.0e-10	-
2	2.5e-13	<2e-16	<2e-16	3.9e=13

Table C.1 Pairwise comparisons of actual dopamine intake. P-values from *post-hoc* pairwise comparisons of mean dopamine doses received in each treatment group (dopamine dose mg/g) using Wilcoxon rank sum tests with correction for multiple testing.

Appendix D

Assessment of L-Dopa dosages received between treatments

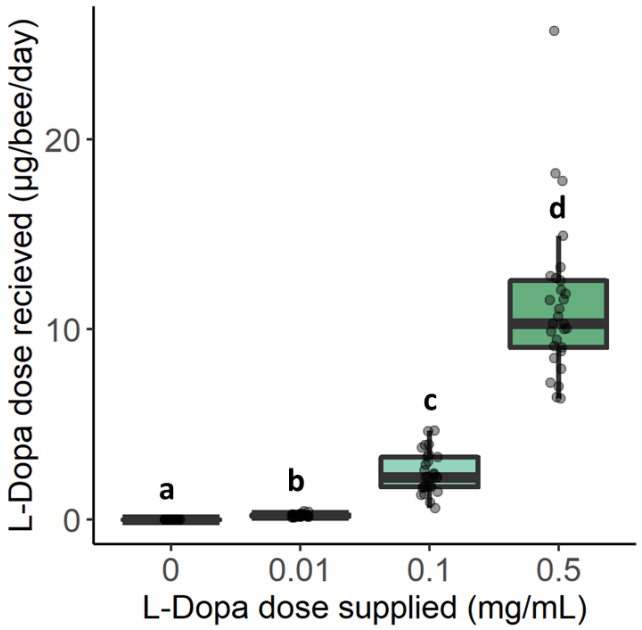

Figure D.1 Actual L-Dopa doses received per bee per day. The actual dopamine doses received in each treatment group were calculated by multiplying the mean water intake (µL/bee/day, where 1 mg water was assumed equivalent to 1 µL) for each treatment by the concentration of L-Dopa supplied in the water. The actual quantity of L-Dopa received differed significantly between treatments (Kruskall-Wallis; $\chi 2 = 109.53$, df = 3, $p < 2.2e\text{-}16$). Pairwise comparisons were made using Wilcoxon rank sum tests with correction for multiple testing (see Table D.1). Statistically significant pairwise comparisons ($p < 0.001$) are denoted by boxplots not sharing a letter.

	0	**0.01**	**0.1**
0.01	5.8e-12	-	-
1	5.8e-12	6.5e-11	-
0.5	5.8e-12	6.5e-11	6.5e-11

Table D.1 Pairwise comparisons of actual L-Dopa intake. P-values from *post-hoc* pairwise comparisons of mean L-Dopa doses received in each treatment group (L-Dopa dose mg/mL) using Wilcoxon rank sum tests with correction for multiple testing.

Appendix E

Assessment of iodotyrosine doses received between treatments

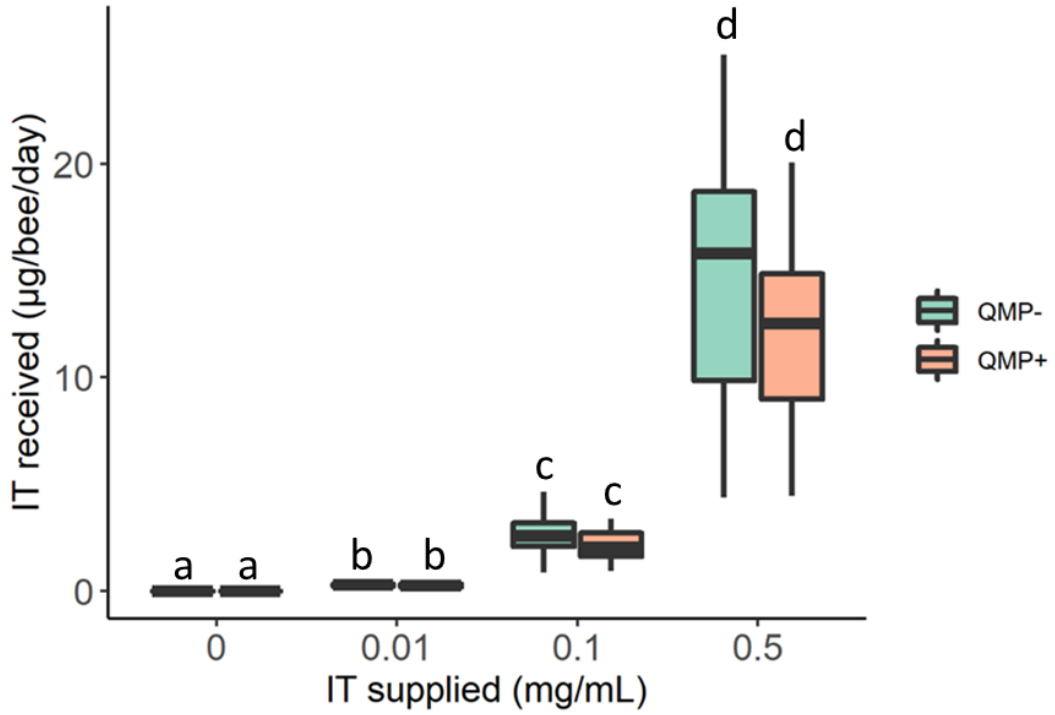

Figure E.1 Actual Iodotyrosine doses received per bee per day. The actual iodotyrosine (IT) doses received differed significantly between each IT treatment group, but not between QMP+ and QMP- treatments. Actual IT doses received in each treatment group were calculated by multiplying the mean water intake (µL/bee/day, where 1 mg water was assumed equivalent to 1 µL) for each treatment by the concentration of IT supplied in the water. Differences in the quantity of IT received between treatment groups were assessed using negative binomial generalised linear mixed effects models (GLMMs). The negative binomial distribution was selected due to zero-inflation of the raw data. IT dose, QMP presence and their interaction were included as fixed effects. To account for repeated measures, day was included as a random effect nested within replicate for all models. The statistical significance of QMP and IT dose supplied on IT dose received was determined by comparing the likelihood ratio of the maximal model to that of the model without the fixed effect of interest. *Post-hoc* pairwise comparisons were made using estimated marginal means on the full GLMM (see Table E.1). Statistically significant pairwise comparisons ($p < 0.001$) are denoted by boxplots not sharing a letter.

Pairwise post-hoc comparisons of actual IT intake between QMP/IT treatments

Contrast	Estimate	SE	df	Z-ratio	P-value
0 0 - 1 0	0.040	0.3976	Inf	0.101	1.0000
0 0 - 0 0.01	-20.437	0.4630	Inf	-44.145	<.0001
0 0 - 1 0.01	-20.328	0.5491	Inf	-37.021	<.0001
0 0 - 0 0.1	-22.713	0.4219	Inf	-53.839	<.0001
0 0 - 1 0.1	-22.503	0.4309	Inf	-52.223	<.0001
0 0 - 0 0.5	-24.474	0.4130	Inf	-59.260	<.0001
0 0 - 1 0.5	-24.292	0.4145	Inf	-58.608	<.0001
1 0 - 0 0.01	-20.477	0.6006	Inf	-34.093	<.0001
1 0 - 1 0.01	-20.368	0.5821	Inf	-34.992	<.0001
1 0 - 0 0.1	-22.753	0.5280	Inf	-43.090	<.0001
1 0 - 1 0.1	-22.543	0.5213	Inf	-43.243	<.0001
1 0 - 0 0.5	-24.514	0.5144	Inf	-47.653	<.0001
1 0 - 1 0.5	-24.332	0.5141	Inf	-47.330	<.0001
0 0.01 - 1 0.01	0.109	0.4308	Inf	0.252	1.0000
0 0.01 - 0 0.1	-2.276	0.3298	Inf	-6.901	<.0001
0 0.01 - 1 0.1	-2.066	0.3376	Inf	-6.120	<.0001
0 0.01 - 0 0.5	-4.037	0.3152	Inf	-12.807	<.0001
0 0.01 - 1 0.5	-3.855	0.3171	Inf	-12.157	<.0001
1 0.01 - 0 0.1	-2.385	0.3652	Inf	-6.530	<.0001
1 0.01 - 1 0.1	-2.175	0.3674	Inf	-5.919	<.0001
1 0.01 - 0 0.5	-4.145	0.3504	Inf	-11.830	<.0001
1 0.01 - 1 0.5	-3.964	0.3502	Inf	-11.321	<.0001
0 0.1 - 1 0.1	0.210	0.1671	Inf	1.256	0.9148
0 0.1 - 0 0.5	-1.761	0.1218	Inf	-14.450	<.0001
0 0.1 - 1 0.5	-1.579	0.1237	Inf	-12.766	<.0001
1 0.1 - 0 0.5	-1.970	0.1337	Inf	-14.735	<.0001
1 0.1 - 1 0.5	-1.789	0.1353	Inf	-13.226	<.0001
0 0.5 - 1 0.5	0.181	0.0696	Inf	2.605	0.1539

Table E.1 Pairwise comparisons of actual iodotyrosine intake. *Post-hoc* pairwise comparisons of actual IT intake between all QMP/IT dose treatment combinations computed using estimated marginal means. “0” encodes QMP- treatments and “1” encodes QMP+ treatments. P-values are Tukey adjusted to control for multiple testing. Note that results are given on the log scale and not the response scale.

Appendix F

Unpublished In Situ Hybridisation data showing *AmDop1* expression in the honey bee ovary

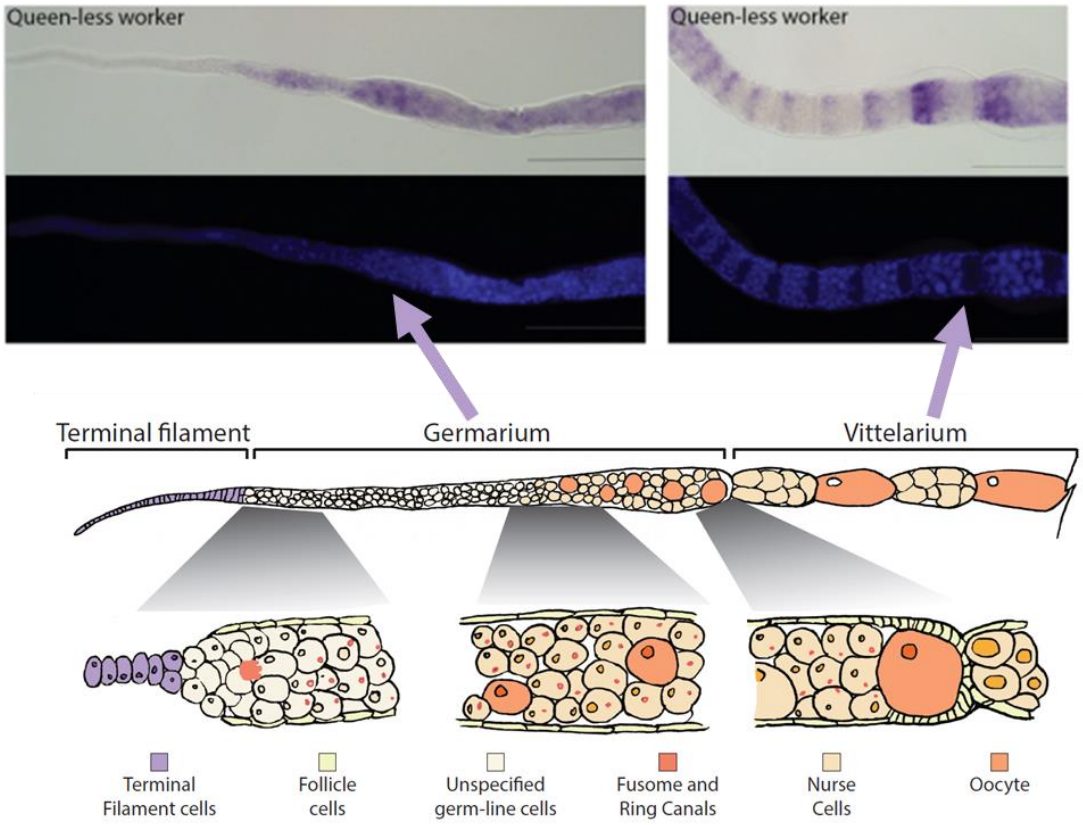

Figure F.1 Expression of *AmDop1* in the honey bee ovary. *In Situ Hybridisation* shows *AmDop1* is expressed in both the germarium and vittelarium. Top: unpublished data, provided by Dr Duncan. Bottom: Figure S11 from Duncan *et al*., 2020, used with permission.

Appendix G

Tables of *post-hoc* pairwise comparisons from Chapter 4

Pairwise Post-hoc comparisons of ovary scores between QMP/iodotyrosine treatments

Contrast	Estimate	SE	df	Z-ratio	P-value
absent 0 - absent 0.1	-1.31433	0.184	Inf	-7.151	<.0001***
absent 0 - present 0.1	-1.45868	0.182	Inf	-8.026	<.0001***
absent 0 - absent 0.5	-2.03199	0.203	Inf	-10.015	<.0001***
absent 0 - present 0.5	-2.14067	0.220	Inf	-9.738	<.0001***
present 0 - absent 0.01	0.83548	0.181	Inf	4.621	0.0001***
present 0 - present 0.01	-0.00969	0.176	Inf	-0.055	1.0000
present 0 - absent 0.1	0.00704	0.180	Inf	0.039	1.0000
present 0 - present 0.1	-0.13731	0.177	Inf	-0.775	0.9944
present 0 - absent 0.5	-0.71061	0.197	Inf	-3.614	0.0073**
present 0 - present 0.5	-0.81929	0.213	Inf	-3.840	0.0031**
absent 0.01 - present 0.01	-0.8456	0.177	Inf	-4.777	<.0001***
absent 0.01 - absent 0.1	-0.82843	0.181	Inf	-4.573	0.0001***
absent 0.01 - present 0.1	-0.97278	0.179	Inf	-5.442	<.0001***
absent 0.01 - absent 0.5	-1.54608	0.199	Inf	-7.755	<.0001***
absent 0.01 - present 0.5	-1.65477	0.216	Inf	-7.657	<.0001***
present 0.01 - absent 0.1	0.01673	0.176	Inf	0.095	1.0000
present 0.01 - present 0.1	-0.12762	0.173	Inf	-0.738	0.9959
present 0.01 - absent 0.5	-0.70092	0.193	Inf	-3.628	0.0069**
present 0.01 - present 0.5	-0.80960	0.210	Inf	-3.847	0.0030**
absent 0.1 - present 0.1	-0.14435	0.177	Inf	-0.813	0.9924
absent 0.1 - absent 0.5	-0.71765	0.197	Inf	-3.638	0.0067**
absent 0.1 - present 0.5	-0.82633	0.214	Inf	-3.858	0.0029**
present 0.1 - absent 0.5	-0.57330	0.194	Inf	-2.952	0.0628
present 0.1 - present 0.5	-0.68198	0.211	Inf	-3.227	0.0274*
absent 0.5 - present 0.5	-0.10868	0.226	Inf	-0.480	0.9997

Table G.1 Ovary scores by QMP/iodotyrosine treatment *Post-hoc* pairwise comparisons of ovary scores between all pairwise comparisons of QMP/ iodotyrosine dose treatment combinations. 'absent' refers to QL microcolonies (QMP-) and 'present' refers to QR microcolonies (QMP+). Iodotyrosine doses are in mg/mL. *Post-hoc* tests were computed using estimated marginal means on the maximal CLMM. P-values are Tukey adjusted.

Pairwise post-hoc comparisons of water intake with QMP/iodotyrosine treatments

Contrast	Estimate	SE	df	T-ratio	P-value
absent 0 - present 0	7.406	1.65	217	4.487	0.0003***
absent 0 - absent 0.01	1.433	1.65	217	0.868	0.9885
absent 0 - present 0.01	4.162	1.65	217	2.522	0.1920
absent 0 - absent 0.1	2.125	1.65	217	1.287	0.9028
absent 0 - present 0.1	7.013	1.65	217	4.249	0.0008***
absent 0 - absent 0.5	-2.087	1.65	217	-1.265	0.9108
absent 0 - present 0.5	2.895	1.65	217	1.754	0.6515
present 0 - absent 0.01	-5.973	1.65	217	-3.619	0.0087**
present 0 - present 0.01	-3.244	1.65	217	-1.966	0.5075
present 0 – absent 0.1	- 5.281	1.65	217	-3.200	0.0333*
present 0 - present 0.1	-0.393	1.65	217	-0.238	1.0000
present 0 - absent 0.5	-9.493	1.65	217	-5.752	<.0001***
present 0 - present 0.5	4.511	1.65	217	-2.733	0.1184
absent 0.01 - present 0.01	2.729	1.65	217	1.653	0.7172
absent 0.01 - absent 0.1	0.692	1.65	217	0.419	0.9999
absent 0.01 - present 0.1	5.580	1.65	217	3.381	0.0191*
absent 0.01 - absent 0.5	-3.520	1.65	217	-2.133	0.3972
absent 0.01 - present 0.5	1.462	1.65	217	0.886	0.9871
present0.01 - absent 0.1	-2.037	1.65	217	-1.234	0.9208
present 0.01 - present 0.1	2.851	1.65	217	1.728	0.6692
present 0.01 - absent 0.5	-6.249	1.65	217	-3.786	0.0048**
present 0.01 - present 0.5	-1.266	1.65	217	-0.767	0.9946
absent 0.1 - present 0.1	4.888	1.65	217	2.962	0.0658
absent 0.1 - absent 0.5	-4.212	1.65	217	-2.552	0.1797
absent 0.1 - present 0.5	0.771	1.65	217	0.467	0.9998
present 0.1 – absent 0.5	-9.100	1.65	217	-5.514	<.0001***
present 0.1 - present 0.5	-4.118	1.65	217	-2.495	0.2032
absent 0.5 - present 0.5	4.983	1.65	217	3.019	0.0562

Table G.2 Water intake by QMP/iodotyrosine treatment *Post-hoc* pairwise comparisons of ovary scores between all pairwise comparisons of QMP/ iodotyrosine dose treatment combinations. 'absent' refers to QL microcolonies (QMP-) and 'present' refers to QR microcolonies (QMP+). Iodotyrosine doses are in mg/mL. *Post-hoc* tests were computed using least squares means on the maximal LMM. P-values are Tukey adjusted.

Pairwise post-hoc comparisons of survival between QMP/iodotyrosine treatments

Contrast	Estimate	SE	df	Z-ratio	P-value
absent 0 - present 0	-1.8264	0.764	Inf	-2.391	0.2454
absent 0 - absent 0.01	-1.2803	0.802	Inf	-1.597	0.7525
absent 0 - present 0.01	-1.7290	0.769	Inf	-2.249	0.3222
absent 0 - absent 0.1	-2.0529	0.753	Inf	-2.727	0.1144
absent 0 - present 0.1	-2.0642	0.753	Inf	-2.742	0.1102
absent 0 - absent 0.5	-4.0030	0.716	Inf	-5.593	<.0001***
absent 0 - present 0.5	-4.2804	0.714	Inf	-5.996	<.0001***
present 0 - absent 0.01	0.5461	0.476	Inf	1.148	0.9460
present 0 - present 0.01	0.0974	0.417	Inf	0.233	1.0000
present 0 - absent 0.1	-0.2266	0.387	Inf	-0.585	0.9991
present 0 - present 0.1	-0.2378	0.387	Inf	-0.614	0.9987
present 0 - absent 0.5	-2.1766	0.309	Inf	-7.043	<.0001***
present 0 - present 0.5	-2.4540	0.305	Inf	-8.048	<.0001***
absent 0.01 – present 0.01	-0.4487	0.483	Inf	-0.928	0.9834
absent 0.01 - absent 0.1	-0.7726	0.458	Inf	-1.688	0.6952
absent 0.01 - present 0.1	-0.7839	0.458	Inf	-1.712	0.6792
absent 0.01 - absent 0.5	-2.7227	0.394	Inf	-6.914	<.0001***
absent 0.01 - present 0.5	-3.0001	0.391	Inf	-7.681	<.0001***
present 0.01 - absent 0.1	-0.3240	0.397	Inf	-0.816	0.9923
present 0.01 - present 0.1	-0.3352	0.397	Inf	-0.844	0.9905
present 0.01 - absent 0.5	-2.2740	0.321	Inf	-7.081	<.0001***
present 0.01 - present 0.5	-2.5514	0.317	Inf	-8.044	<.0001***
absent 0.1 - present 0.1	-0.0112	0.365	Inf	-0.031	1.0000
absent 0.1 - absent 0.5	-1.9501	0.281	Inf	-6.945	<.0001***
absent 0.1 – present 0.5	-2.2274	0.276	Inf	-8.063	<.0001***
present 0.1 - absent 0.5	-1.9388	0.281	Inf	-6.904	<.0001***
present 0.1 - present 0.5	-2.2162	0.276	Inf	-8.022	<.0001***
absent 0.5 - present 0.5	-0.2774	0.147	Inf	-1.891	0.5574

Table G.3 Survival by QMP/iodotyrosine treatment *Post-hoc* pairwise comparisons of ovary scores between all pairwise comparisons of QMP/ iodotyrosine dose treatment combinations. ‘absent’ refers to QL microcolonies (QMP-) and ‘present’ refers to QR microcolonies (QMP+). Iodotyrosine doses are in mg/mL. *Post-hoc* tests were computed using least squares means on the maximal CPH. P-values are Tukey adjusted.

Appendix H

RT-qPCR

Table H.1 Oligonucleotide sequences used for RT-qPCR

Target gene	**HoneybeeID***	**5' primer**	**3' primer**
Oligonucleotide sequences used to detect expression of reference genes			
Rnp2 26S Proteasome non-ATPase regulatory subunit 1	GB52526	CGC CTG TAA TGG AAA CTG AAA	ACA CGT TCT TGT TGC TCA CG
Oligonucleotide sequences used to detect expression of target genes			
bHLH2	GB43790	GGG AAG CGG GAT CAA GAT A	AGT CTG GGC GAG GAG ATG TA
Her	GB43788	ACC ACC ACC GTA GCA TCA TC	ACT TTG GGG AGG CGT GTA A

* gene identifiers are from BeeBase genome assembly v4.5 official gene set version 3.2

Table H.2 n values for each QMP dose/ ovary score combination

QMP Dose (QE/µL)	Ovary score	N
0	0	3
	1	3
	2	3
	3	3
0.01	0	0
	1	3
	2	3
	3	0
0.1	0	3
	1	3
	2	3
	3	1
1	0	0
	1	3
	2	3
	3	3

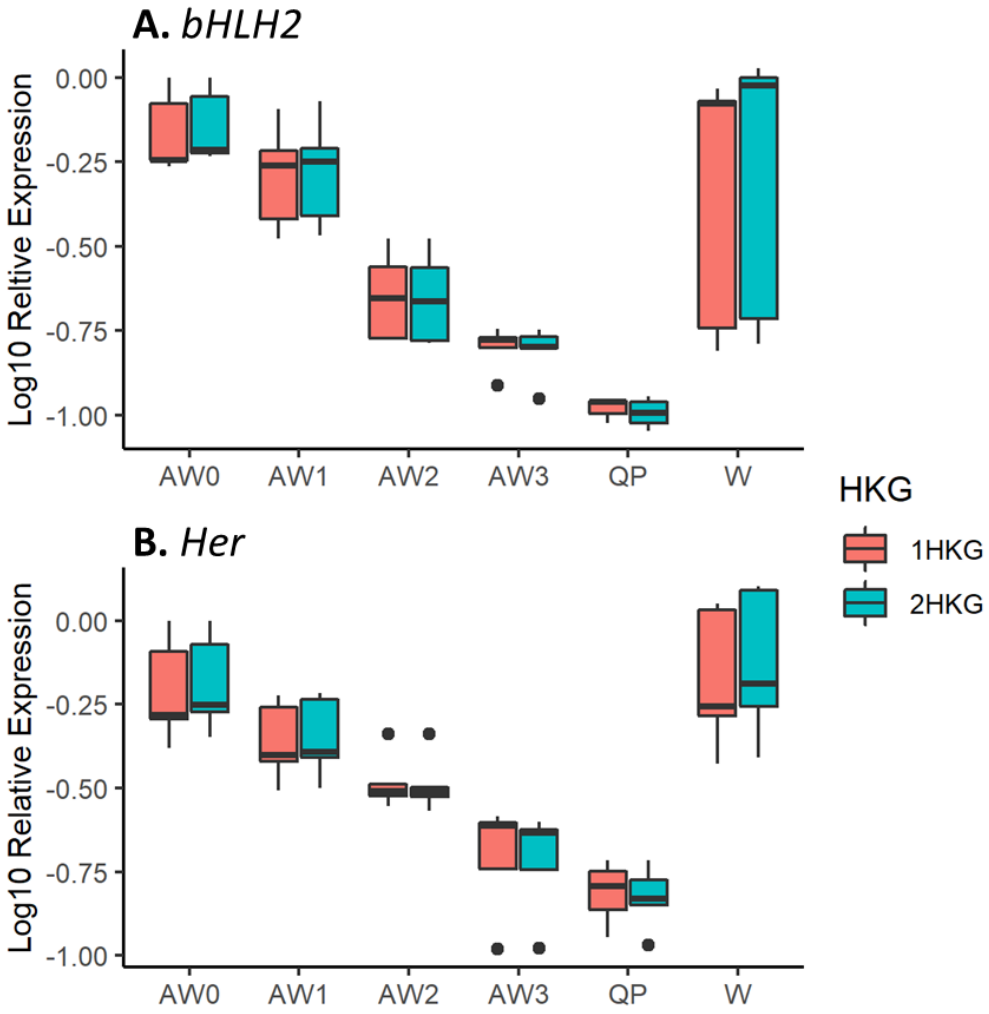

Figure H.1 Validation of single reference gene usage Past gene expression data measured using RT-qPCR of the genes **A.** *bHLH2* and **B.** *Her* in worker ovaries in different worker groups. Raw data from (Duncan *et al*., 2016) was provided by E.J. Duncan and used with permission. Relative expression was normalized to expression levels of just one housekeeping gene as reference (*Rpn2;* "1HKG", red boxplots) or the geometric mean of expression levels of *Rpn2* and a second housekeeper, *mRPL44* ("2HKG", blue boxplots). Differences in expression of each gene between groups were assessed using ANOVA. To determine whether the number of housekeeping genes used would have altered experimental outcomes, each analyses was carried out on expression data normalized to one vs two housekeeping genes (1HKG vs 2HKG). The number of housekeepers used for normalization had a subtle effect on effect sizes, but was ultimately inconsequential to the results of statistical analyses. *bHLH2* expression differed significantly between treatment groups whether normalized to 1HKG ($F(5) = 8.20$, $p<0.001$) or 2HKG ($F(5) = 7.96$, $p<0.001$). *Her* expression also was significantly different between treatment groups whether normalized to 1HKG ($F(5) = 8.00$, $p<0.001$) or 2HKG ($F(5) = 8.62$, $p<0.001$). The use of *Rpn2* only as a reference gene for RT-qPCR is therefore deemed to be appropriate.

Appendix I

Validation of QMP efficacy experiment two

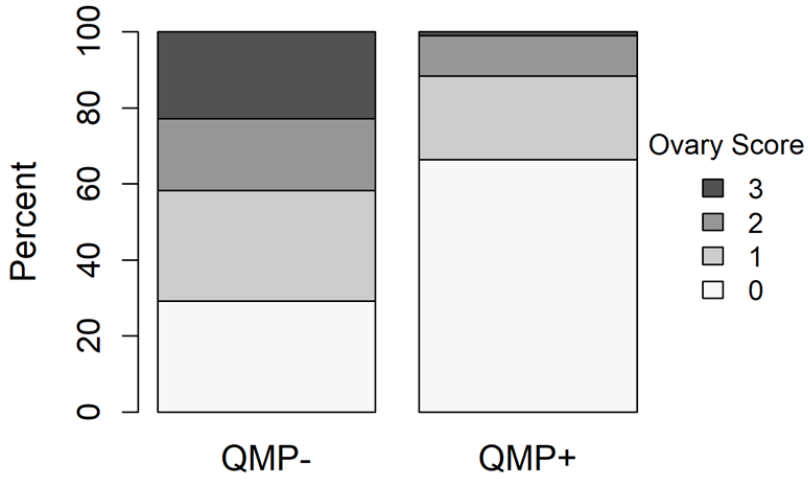

Figure I.1 Validation of QMP's repression of ovary activity in experiment two. A subset of bees from +/- QMP control (0ppb IMI) cages were dissected to verify that QMP was causing the expected ovary repression phenotype. 100 ovaries were scored from each treatment across 3 reps (40 from rep 1, 30 from rep 2, 30 from rep 3). Ovaries were scored using blinder software. A Chi-squared test was performed in R to test whether the proportions of each ovary score differed with QMP treatment. QMP treatment effectively reduced ovary development (Pearson's Chi Squared: $\chi 2 = 35.92$, $df = 3$, $p < 0.0001$), validating the efficacy of QMP treatments in experiment 2.

Appendix J

Imidacloprid sublethal doses preliminary study

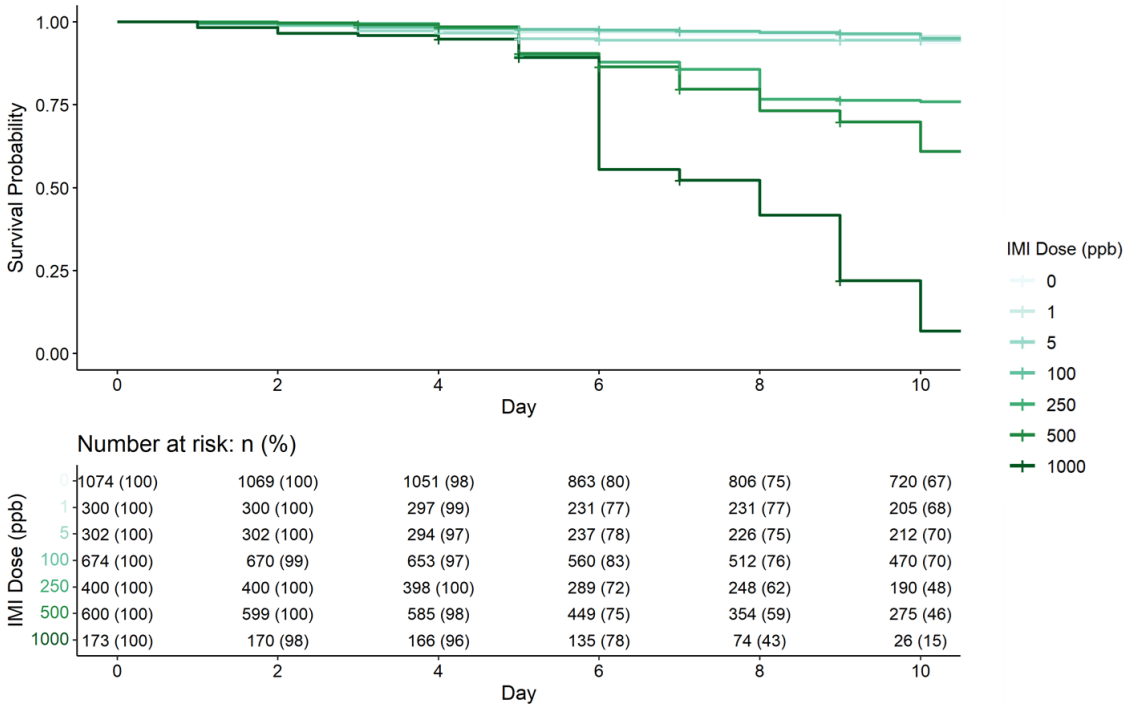

Figure J.1 Assessment of imidacloprid lethal dosage in laboratory microcolonies. Survival data from a preliminary study assessing the effect of different imidacloprid doses on mortality in our laboratory setup over ten days. Data shown is pooled across multiple replicates for each imidacloprid dose. Cages were setup to initially contain 70-100 workers. Survival data is shown as Kaplan Meier survival distribution curves with a risk table showing absolute numbers of bees (n) surviving to a given timepoint. Percentages of bees (%) surviving is also displayed for ease of comparison between different imidacloprid doses. An imidacloprid dose of 250 ppb led to the death of 52% of bees by day ten, making this dose our best estimate of the LD50 using the administration methodology in this experimental setup. Survival was markedly improved at doses of 100 ppb and bellow, in which survival rates were approximately 70% by day ten, not dissimilar from the survival rates generally observed in the laboratory (personal observation) or from 0 ppb controls. Imidacloprid doses bellow 100 ppb were thus considered to be 'sub-lethal' for this experiment.

Appendix K

Tables of *post-hoc* pairwise comparisons from Chapter six

Experiment One – Solution Intake					
Contrast	**Estimate**	**SE**	**Df**	**T-ratio**	**P-value**
QMP- 0 ppb – QMP+ 0 ppb	7.93	2.16	138	3.67	0.0046**
QMP- 0 ppb – QMP- 1 ppb	2.79	2.18	138	1.28	0.7966
QMP- 0 ppb – QMP+ 1 ppb	7.11	2.16	137	3.29	0.0157*
QMP- 0 ppb – QMP- 5 ppb	1.48	2.23	139	0.66	0.9855
QMP- 0 ppb – QMP+ 5 ppb	7.81	2.23	138	3.50	0.0082**
QMP+ 0 ppb – QMP- 1 ppb	-5.13	2.19	138	-2.35	0.1819
QMP+ 0 ppb – QMP+ 1 ppb	-0.82	2.17	138	-0.38	0.9990
QMP+ 0 ppb – QMP- 5 ppb	-6.45	2.22	138	-2.90	0.0485*
QMP+ 0 ppb – QMP+ 5 ppb	-0.12	2.25	139	-0.05	1.0000
QMP- 1 ppb – QMP+ 1 ppb	4.32	2.18	137	1.99	0.3560
QMP- 1 ppb – QMP- 5 ppb	-1.31	2.23	138	-0.59	0.9918
QMP- 1 ppb – QMP+ 5 ppb	5.02	2.26	138	2.22	0.2354
QMP+ 1 ppb – QMP- 5 ppb	-5.63	2.21	138	-2.55	0.1181
QMP+ 1 ppb – QMP+ 5 ppb	0.70	2.24	138	0.31	0.9996
QMP- 5 ppb – QMP+ 5 ppb	6.33	2.31	140	2.75	0.0730
Experiment Two – Solution Intake					
Contrast	**Estimate**	**SE**	**Df**	**T-ratio**	**P-value**
QMP- 0 ppb – QMP+ 0 ppb	7.69	1.67	151	4.60	0.0001***
QMP- 0 ppb – QMP- 1 ppb	-1.06	1.67	151	-0.64	0.9882
QMP- 0 ppb – QMP+ 1 ppb	6.46	1.65	151	3.91	0.0019**
QMP- 0 ppb – QMP- 5 ppb	-0.97	1.65	151	-0.59	0.9918
QMP- 0 ppb – QMP+ 5 ppb	5.67	1.67	151	3.40	0.0111*
QMP+ 0 ppb – QMP- 1 ppb	-8.75	1.67	151	-5.24	<.0001***
QMP+ 0 ppb – QMP+ 1 ppb	-1.22	1.65	151	-0.74	0.98
QMP+ 0 ppb – QMP- 5 ppb	-8.65	1.65	151	-5.24	<.0001***

QMP+ 0 ppb – QMP+ 5 ppb	-2.02	1.67	151	-1.21	0.8332
QMP- 1 ppb – QMP+ 1 ppb	7.52	1.65	151	4.55	0.0002***
QMP- 1 ppb – QMP- 5 ppb	0.09	1.65	151	0.05	1.0000
QMP- 1 ppb – QMP+ 5 ppb	6.73	1.67	151	4.03	0.0012**
QMP+ 1 ppb – QMP- 5 ppb	-7.44	1.64	151	-4.54	0.0002***
QMP+ 1 ppb – QMP+ 5 ppb	-0.79	1.65	151	-0.48	0.9968
QMP- 5 ppb – QMP+ 5 ppb	6.64	1.65	151	4.02	0.0013**
Experiment Two – Food Intake					
Contrast	**Estimate**	**SE**	**Df**	**T-ratio**	**P-value**
QMP- 0 ppb – QMP+ 0 ppb	-1.80	0.35	154	-5.12	<0.0001***
QMP- 0 ppb – QMP- 1 ppb	-0.04	0.35	154	-0.11	1.0000
QMP- 0 ppb – QMP+ 1 ppb	-1.13	0.35	154	-3.24	0.0181*
QMP- 0 ppb – QMP- 5 ppb	0.47	0.35	154	1.36	0.7531
QMP- 0 ppb – QMP+ 5 ppb	-0.86	0.35	154	-2.46	0.1443
QMP+ 0 ppb – QMP- 1 ppb	1.76	0.35	154	5.01	<0.0001***
QMP+ 0 ppb – QMP+ 1 ppb	0.67	0.35	154	1.91	0.4002
QMP+ 0 ppb – QMP- 5 ppb	2.27	0.35	154	6.46	<0.001***
QMP+ 0 ppb – QMP+ 5 ppb	0.944	0.35	154	2.69	0.0841
QMP- 1 ppb – QMP+ 1 ppb	-1.09	0.35	154	-3.13	0.0251*
QMP- 1 ppb – QMP- 5 ppb	0.51	0.35	154	1.47	0.6870
QMP- 1 ppb – QMP+ 5 ppb	-0.82	0.35	154	-2.35	0.1824
QMP+ 1 ppb – QMP- 5 ppb	1.60	0.35	154	4.59	0.0001***
QMP+ 1 ppb – QMP+ 5 ppb	0.27	0.35	154	0.78	0.9700
QMP- 5 ppb – QMP+ 5 ppb	-1.33	0.35	154	-3.811	0.0027**

Table K.1 Pairwise *post-hoc* comparisons for food and solution intake in experiments one and two between all QMP and imidacloprid treatment combinations computed using estimated marginal means on the maximal LMM. P-values are Tukey adjusted.

Brian Dopamine					
Contrast	**Estimate**	**SE**	**Df**	**Z-ratio**	**P-value**
QMP- 0 ppb – QMP+ 0 ppb	-1.05e-04	0.000210	Inf	-0.49	0.99
QMP- 0 ppb – QMP- 1 ppb	2.44e-04	0.000359	Inf	0.68	0.98
QMP- 0 ppb – QMP+ 1 ppb	1.68e-04	0.000359	Inf	0.47	0.99
QMP- 0 ppb – QMP- 5 ppb	2.18e-04	0.000185	Inf	1.17	0.85
QMP- 0 ppb – QMP+ 5 ppb	-6.44e-04	0.000238	Inf	-2.70	0.07
QMP+ 0 ppb – QMP- 1 ppb	3.49e-04	0.000350	Inf	1.00	0.91
QMP+ 0 ppb – QMP+ 1 ppb	2.72e-04	0.000349	Inf	0.79	0.97
QMP+ 0 ppb – QMP- 5 ppb	3.23e-04	0.000187	Inf	1.73	0.51
QMP+ 0 ppb – QMP+ 5 ppb	-5.39e-04	0.000246	Inf	-2.19	0.24
QMP- 1 ppb – QMP+ 1 ppb	-7.63e-05	0.000251	Inf	-0.30	0.99
QMP- 1 ppb – QMP- 5 ppb	-2.59e-05	0.000346	Inf	-0.08	1.00
QMP- 1 ppb – QMP+ 5 ppb	-8.88e-04	0.000383	Inf	-2.34	0.18
QMP+ 1 ppb – QMP- 5 ppb	5.04e-05	0.000346	Inf	0.15	1.00
QMP+ 1 ppb – QMP+ 5 ppb	-8.11e-04	0.000382	Inf	-2.14	0.27
QMP- 5 ppb – QMP+ 5 ppb	-8.62e-04	0.000225	Inf	-3.84	0.0017**

Table K.2 Pairwise *post-hoc* comparisons of brain dopamine levels between all QMP and imidacloprid treatment combinations computed using estimated marginal means on the maximal GLMM. GLMM was fitted with an inverse link function, hence contrast estimates are still on the inverse scale. P-values are Tukey adjusted.

Experiment One Survival					
Contrast	**Estimate**	**SE**	**Df**	**Z-ratio**	**P-value**
QMP- 0 ppb – QMP+ 0 ppb	-0.22	0.27	Inf	-0.82	0.96
QMP- 0 ppb – QMP- 1 ppb	0.76	0.35	Inf	2.17	0.25
QMP- 0 ppb – QMP+ 1 ppb	0.70	0.25	Inf	-2.85	<0.05*
QMP- 0 ppb – QMP- 5 ppb	0.36	0.31	Inf	1.18	0.85
QMP- 0 ppb – QMP+ 5 ppb	0.71	0.27	Inf	-2.62	0.09
QMP+ 0 ppb – QMP- 1 ppb	0.98	0.34	Inf	2.89	<0.05*
QMP+ 0 ppb – QMP+ 1 ppb	-0.48	0.23	Inf	-2.10	0.29
QMP+ 0 ppb – QMP- 5 ppb	0.58	0.30	Inf	1.97	0.36
QMP+ 0 ppb – QMP+ 5 ppb	0.49	0.26	Inf	-1.91	0.39
QMP- 1 ppb – QMP+ 1 ppb	-1.47	0.32	Inf	-4.54	<0.001***
QMP- 1 ppb – QMP- 5 ppb	-0.40	0.37	Inf	-1.07	0.89
QMP- 1 ppb – QMP+ 5 ppb	-1.47	0.34	Inf	-4.31	<0.001***
QMP+ 1 ppb – QMP- 5 ppb	1.07	0.28	Inf	3.86	<0.01**
QMP+ 1 ppb – QMP+ 5 ppb	-0.01	0.23	Inf	-0.021	1.00
QMP- 5 ppb – QMP+ 5 ppb	-1.07	0.30	Inf	-3.60	<0.01**
Experiment Two Survival					
Contrast	**Estimate**	**SE**	**Df**	**Z-ratio**	**P-value**
QMP- 0 ppb – QMP+ 0 ppb	-1.21	0.21	Inf	-5.87	<0.001***
QMP- 0 ppb – QMP- 1 ppb	-0.24	0.24	Inf	-1.01	0.92
QMP- 0 ppb – QMP+ 1 ppb	-0.23	0.24	Inf	-0.95	0.93
QMP- 0 ppb – QMP- 5 ppb	-0.13	0.34	Inf	-0.56	0.99
QMP- 0 ppb – QMP+ 5 ppb	-0.95	0.21	Inf	-4.53	<0.001***
QMP+ 0 ppb – QMP- 1 ppb	0.97	0.19	Inf	5.16	<0.001***
QMP+ 0 ppb – QMP+ 1 ppb	0.98	0.19	Inf	5.17	<0.001***
QMP+ 0 ppb – QMP- 5 ppb	1.07	0.20	Inf	5.50	<0.001***
QMP+ 0 ppb – QMP+ 5 ppb	0.25	0.16	Inf	1.63	0.58
QMP- 1 ppb – QMP+ 1 ppb	0.01	0.22	Inf	0.05	1.00
QMP- 1 ppb – QMP- 5 ppb	0.10	0.23	Inf	0.45	1.00
QMP- 1 ppb – QMP+ 5 ppb	-0.72	0.19	Inf	-3.70	<0.01**
QMP+ 1 ppb – QMP- 5 ppb	0.09	0.23	Inf	0.40	1.00
QMP+ 1 ppb – QMP+ 5 ppb	-0.73	0.20	Inf	-3.73	<0.01**
QMP- 5 ppb – QMP+ 5 ppb	-0.82	0.20	Inf	-4.10	<0.001***

Table K.3 – Pairwise *post-hoc* comparisons of survival distributions between all QMP and imidacloprid treatment combinations computed using estimated marginal means on the maximal CPH model with mixed effects.

Appendix L

Vitellogenin expression unpublished data

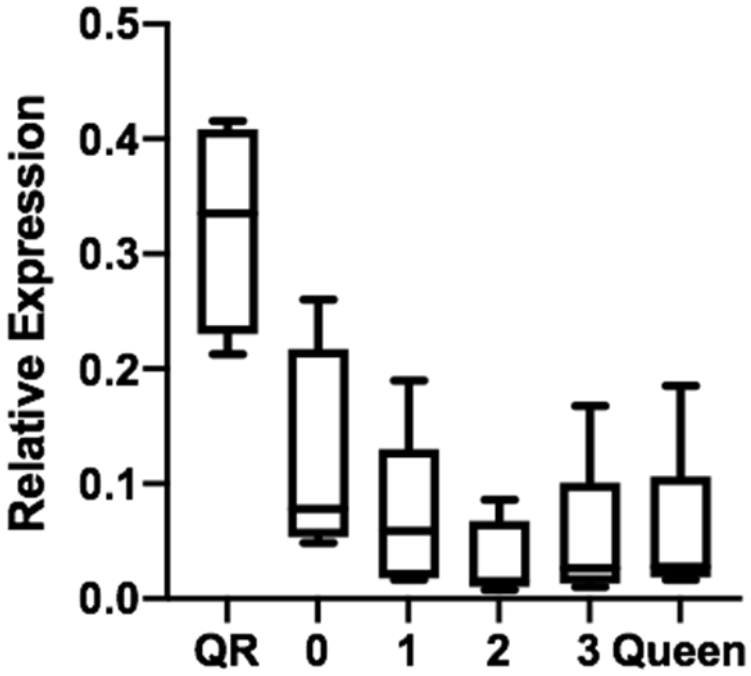

Figure L.1 Ovary expression of *vitellogenin* measured using RT-qPCR. *Vitellogenin* expression was measured in QR workers, QL workers with differing degrees of ovary activity, and Queens. Expression of *vitellogenin* is reduced in QL relative to QR workers, even where there are no changes in ovary morphology (i.e. scores of 0 and 1). Unpublished data provided by Dr Duncan.

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