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Tracking behavioural and neuronal responses to social pheromones: Insights from a Drosophila model

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ABSTRACT

If eusociality evolved through modification of pre-social mechanisms for regulating personal reproduction, then even insects like *Drosophila* may be vulnerable to latent effects of 'queen' pheromone. Here, I test if male fruit flies respond to a eusocial queen bee pheromone. I found that male flies were attracted to queen bee pheromone, and pheromone-treated males raised the intensity of their courting towards conspecific females. These novel observations from *Drosophila* suggest that male flies have the capacity to respond to queen pheromone in a manner that is comparable to the native response from male (drone) bees. I therefore optimized a nuclear factor of activated T-cell (NFAT) system to label olfactory neurons that are putatively responsive to the pro-reproductive pheromone. The NFAT reporter system implicates three neurons (Or-49b, Or-56a, Or-98a) that, if shown to function similarly in drones, will validate my use of *Drosophila* to probe otherwise unknown mechanisms of social bee communication.

Keywords

Drosophila melanogaster; *Apis mellifera*; T-maze; NFAT; social evolution; comparative analysis; queen pheromones; queen mandibular pheromone; ground plan hypothesis

CO-AUTHORSHIP STATEMENT

Chapter 2: Sexual response of male *Drosophila* to honey bee queen mandibular pheromone: implications for genetic studies of social insects was written by Justin Croft with Dr. Graham Thompson, Tom Liu, Dr. Alison Camiletti and Dr. Anne Simon as co-authors. Specifically, Justin Croft conducted all T-maze experiments, performed subsequent statistical analysis on the data, and co-wrote the manuscript. Tom Liu conducted all mating experiments and performed subsequent statistical analysis. Dr. Graham Thompson, Dr. Anne Simon and Dr. Alison Camiletti helped to conceive the idea, and provided experimental oversight. All authors approved the manuscript prior to submission for publication.

Chapter 3: Mapping neuronal responses to a social pheromone in *Drosophila melanogaster* was written by Justin Croft with Dr. Graham Thompson and Dr. Alison Camiletti as co-authors. Specifically, Justin Croft conducted all experiments, performed confocal image analysis, performed subsequent statistical analysis on the data, and wrote the manuscript. Dr. Graham Thompson and Dr. Alison Camiletti helped to conceive the idea and provided experimental oversight. Dr. Graham Thompson edited the manuscript. I intend to submit this as a manuscript for publication.

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LIST OF ABBREVIATIONS

9-HDA: 9-hydroxydec-2-enoic acid

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

***Am*Or**: *Apis mellifera* olfactory receptor

ANOVA: analysis of variance

BSC ID: Bloomington Stock Center identification number

CaLexA: calcium-dependent nuclear import of LexA

CI: confidence interval

CRISPR: Clustered regularly interspaced short palindromic repeats

D-PBS: Dulbecco's Phosphate-buffered saline

FET: Fishers exact test

for: *foraging*

***for*R**: *foraging* rover allele

***for*S**: *foraging* sitter allele

GAL4: yeast transcription activator protein

GFP: Green fluorescent protein

GPH: Ground Plan Hypothesis

HOB: Methyl p-hydroxybenzoate

HVA: 4-hydroxy-3-methoxyphenylethanol or homovanillyl alcohol

NFAT: nuclear factor of activated T-cells

Or: olfactory receptor

ORCO: Odorant receptor co-receptor

Ore-R: *Oregon-R*

PBT: Phosphate buffered saline with Triton-X

PI: Preference index

Qqe: queen equivalent

QMP: queen mandibular pheromone

RNAi: RNA interference

TALEN: Transcription activator-like effector nucleases

UAS: Upstream Activation Sequence

w^{1118}: mutation on each X chromosome, which gives a fly white eyes

ZNF: Zinc-finger nucleases

Chapter 1

1 General introduction

1.1 From pre-social to eusocial breeding systems

Eusocial insect colonies function as social units in which groups of individuals execute specific jobs, to the exclusion of others. The principle division of labour is between reproductive and non-reproductive castes (Wilson and Hölldobler 2005). Division of reproductive labour is a hallmark of eusocial breeding systems, and in some species this division is so pronounced that individuals are effectively dependent on one another for survival and even for reproduction (Cervo 2006; Seeley 1995). This evolutionary integration of reproductive interests is curious because of the reproductive altruism that evolves among the non-reproductive castes (Bourke 2014). The evolution of altruism is ultimately explained by inclusive fitness theory (Hamilton 1964; Bourke 2011; Marshall 2015), which shows - at least mathematically - how reproductive helping can evolve if it is directed towards reproducing kin (Gardner et al. 2011). Thus, kinship can align the reproductive interests of different castes, resulting in a highly cooperative division of labour between sub-fertile helpers and their reproducing relatives.

At the same time, differences in kinship and reproductive potential among colony mates can promote conflict over cooperation, as individuals pit their reproductive

interests against one another (Ratnieks et al. 2006). The balance between cooperation and conflict within insect societies is dependent on changes to social circumstance that may alter the fitness pay-off of one group of individuals versus another (Strassmann et al. 2011). For example, starved cells of a social amoeba, *Dictyostelium discoideum*, will come together in large groups in which some cells sacrifice themselves to make a stalk that supports in the dispersal of others as spores (Kessin 2000). This social tension within colonies is mediated through real-time communication of differences in kinship and reproductive potential (Bourke 1999). That is, the ultimate fitness consequences of social living are mediated by proximate mechanisms, such as DNA methylation or histone modification, which have evolved from pre-social ancestries (Crespi and Choe 1997). For many social insects, the main form of communication is pheromones (Le Conte and Hefetz 2008), though other modes of communication (e.g. vibration, cuticular hydrocarbons, etc.) are important (Hunt and Richard 2013; van Zweden and d'Ettorre 2010). Pheromones as signals, and the mechanisms that receive them, are therefore likely to co-evolve with sociality, and studying these mechanisms may provide insights into the social origins of certain insect groups.

1.2 *Apis* as a model for identifying genes associated with eusociality

Eusociality is considered to be the most complex form of sociality and is defined as; cooperative care of offspring, a division of labour consisting of reproductive and non-

reproductive individuals, and an overlap of generations capable of reproduction (Crespi and Yanega 1995). This extreme form of social breeding has evolved repeatedly among insects, including in termites (Noirot 1990), some species of beetles (Kent and Simpson 1992) and aphids (Aoki 1977), as well as occasionally among other Arthropods (Duffy and Thiel 2007) and mammals (Clarke and Faulkes 1997). Among insects the order Hymenoptera is the largest and most well-known animal group with eusocial species. Most Hymenoptera are not eusocial, but this social system has evolved at least eleven times independently within that order (Hughes et al. 2008; Johnson et al. 2013). As such, the genes and pathways that influence eusocial traits have been best studied using eusocial species of bees, ants and wasps (Yan et al. 2014).

Honey bee societies do lend themselves well to genomic analysis. Not only are they eusocial, but owing to their agricultural importance, are well understood in terms of colony structure, and can be manipulated to create social phenotypes of interest (e.g., queenright versus queenless colonies, or workers all the same age, etc.). Within honey bee colonies the principal form of communication is via queen and brood pheromones (Bortolotti and Costa 2014). The queen mandibular pheromone (QMP), in particular, is well characterised (Hoover et al. 2003; Strauss et al. 2008; Pankiw et al. 2000), and is thought to have evolved to serve multiple fitness-related roles (Slessor et al. 2005).

First, QMP signals fertility. On mating flights, a virgin queen emits QMP to attract male (drone) bees to mate with her (Gary and Marston 1971). This functional role of queen pheromone suggests that QMP likely evolved from pre-social fertility signals to attract males (Van Oystaeyen et al. 2014; Oi et al. 2015). Second, queen mandibular pheromone signals fecundity. Within colonies, mated queens emit QMP to signal egg-laying potential (Kocher and Grozinger 2011). Workers respond to this evolutionarily honest signal by having their ovaries de-activated and otherwise adopting a reproductively altruistic role within the colony (Amdam et al. 2006). This latter role for queen pheromone as a social signal has likely co-evolved with eusociality itself (Chapuisat 2014; Oi et al. 2015) but may have pre-social antecedents, as evidenced by its latent effect on some pre-social taxa (Sannasi 1969).

In 2006, the honey bee, *Apis mellifera* was the first eusocial insect to feature a draft genome assembly (Weinstock et al. 2006). Subsequently, high-quality draft genomes of ant species (Nygaard et al. 2011; Smith et al. 2011), other bee species (Chen et al. 2013; Sadd et al. 2015) and several other eusocial and subsocial species have become available in the last few years (Kapheim 2016). These social insect genomes have accelerated the field of 'sociogenomics' (Sumner 2014) and, for the first time, we have been able to ask: are there common genomic features that underpin different instances of social evolution? Thus far comparative studies have found that, indeed, common pathways may regulate some eusocial traits, like worker

sterility and worker foraging behavior (Toth and Rehan 2016). For example, the termite *Cryptotermes secundus* may use a similar gene network (vitellogenin-like) to regulate female egg-laying as does the honey bee (Weil et al. 2007). Additionally, genes involved in pathways associated with development and metabolism may regulate worker caste differences in separate species of ants (Mikheyev and Linksvayer 2015), as well as in *Apis* (Mutti et al. 2011). Within the genus *Apis*, analysis of microarray studies has suggested that insulin signaling (Mullen et al. 2014), dopamine (Oxley et al. 2008) and target of rapamycin (TOR; Cardoen et al. 2011) pathways may be implicated as being functionally associated with honey bee division of reproductive labour and worker sterility.

1.3 The need for a genetically tractable model in sociobiology

While these types of comparative analyses continue to implicate a growing list of candidate genes and pathways that underlie variation in eusocial traits (Toth and Rehan 2016), the function of most genes are rarely tested *in vivo*. This apparent lag in functional genetic studies in sociobiology may in part be due to limitations from social Hymenoptera as a model system (Camiletti and Thompson 2016). The Western honey bee, *Apis mellifera* may be the most advanced model. Recent investment into this species as a partially tractable genetic model has seen the development of transgenic bees via germ-line transformation. Schulte et al. (2014) showed that a *piggyBac*-derived cassette can be used to knock-out a target gene by

inserting into the coding region and disrupting translation. The transgenic vector was stably transmitted into queens (a 20-27% success rate), and subsequently incorporated into recombinant offspring (workers). This pioneering technique, therefore, offers the prospect of manipulating gene function *in vivo*. Utilizing germ-line targeted transposons like this may soon make it feasible for a fully pliable gene expression system within *Apis* that is comparable to the GAL4/UAS system that has long been available for *Drosophila* (Ben-Shahar 2014).

Further, direct genome editing technologies like ZFNs (Bibikova et al. 2002), TALENs (Joung and Sander 2013) and CRISPR (Cong et al. 2013) are now regularly being used to manipulate the genomes of model mice, *Drosophila*, nematodes and *Arabidopsis* (Doudna and Charpentier 2014), and may in future become feasible for a wider array of non-model species. If so, it may become possible to edit the genome of the eusocial honeybee (Reid and O'Brochta 2016). An initial study by Kohno et al. (2016) used CRISPR to transform a singular queen that produced genome-edited male offspring at a rate of 12.5% that contained the specific gene knockout. These technologies, while still in their infancy in social insects, could pave the way for future functional analyses of candidate genes involved in honeybee social behaviors.

Despite this progress, manipulation of bee genomes is not yet widely feasible. RNAi technology nonetheless provides one practical opportunity to test candidate genes

for honeybee social behavior (Amdam et al. 2003). RNAi-gene specific knockdown analysis provides a powerful tool to study phenotypic effects associated with gene knockdowns but is susceptible to technical failure and non-specific effects (Summerton 2007; Jarosch and Moritz 2011; Scott et al. 2013). An alternative approach might be to adopt a non-social but genetically tractable model to test basic predictions about the molecular mechanisms governing social or non-social reproduction (Camiletti and Thompson 2016).

1.4 *Drosophila* as a behavioural and neuronal model for *Apis*

Despite limits to gene manipulation techniques that are currently applicable for eusocial taxa, it may be possible to exploit the most powerful techniques now by using a surrogate taxon for which the technology is already optimised. *Drosophila* is not a eusocial organism, but has been shown effective at uncovering conserved genetic mechanisms underpinning response to queen pheromone and reproductive regulation (Camiletti and Thompson 2016). Within our lab, Camiletti et al. (2013) showed that female *Drosophila melanogaster* exposed to honey bee queen mandibular pheromone had smaller ovaries with fewer mature eggs than did untreated controls. This response from a pre-social insect is not without precedent (Sannasi 1969) but is noteworthy as female flies apparently de-activate their ovaries as if they were partially sterile, which mimics the response of the worker bee herself.

Camiletti et al. (2014) extended this bee-fly comparison to show that *sitter/rover* lines of *Drosophila* (Sokolowski 2001) are differentially responsive to QMP, as are the nurse/forager sub-castes of honey bee workers (Fussnecker et al. 2011). *Sitters* exposed to queen pheromone respond as nurse-age workers typically do, by de-activating their ovaries. *Rovers*, by contrast, were less responsive to the pheromone, much like forager-age workers. These results emphasize an interspecific effect of a social cue on a phylogenetically distant pre-social taxon.

Since olfaction is likely to be the initial step in any downstream pheromonal effect (Carcaud et al. 2015), Camiletti et al. (2016) screened a majority (75%) of the 60 olfactory receptors (Or) present in *Drosophila*, and identified several that partially prevented the anti-ovarian response to queen pheromone. From this assay, a short candidate list of receptors (Or-49b, Or-56a and Or-98a) was identified that may be responsible for the peculiar response of female flies to QMP. Further, the structural similarity of candidate olfactory receptor binding domains was compared to the five main components of QMP (9-ODA, HOB, HVA and +/- 9-HDA) for ligand-molecule binding similarity. Here, each candidate olfactory receptor predicted biological affinity as a ligand for different components of queen mandibular pheromone suggesting bees themselves may also use several olfactory receptors to perceive and respond to QMP.

Further evidence for behavioural-genetic homology between *Apis* and *Drosophila* can be found in studies that apply other bee-derived social signals to this pre-social insect to observe a bee-like response. Kamakura (2011), for instance, used royalactin, a component of royal jelly (Kamakura 2002), to elicit similar bee-like phenotypes in *Drosophila*. Young fly larvae reared in a royalactin-based medium developed faster, developed into larger female flies, and developed larger ovaries. Each of these three *Drosophila* responses to royalactin suggest a degree of conservation with honey bee larvae involved in worker-to-queen transitions with royal jelly. The effects of these social cues (i.e., royal jelly and QMP) in *Drosophila* are consistent with the hypothesis that social and pre-social species can remain functionally conserved with respect to mechanisms that regulate physiological traits.

These observations raise the question: is it possible that *Apis* and *Drosophila* can 'communicate' via a conserved pheromone-responsive pathway that regulates both behaviour and physiology? If so, it may be possible to use the fully tractable fly as a model to dissect this response and reveal the specific genes and neural circuits involved, even if they are imperfectly conserved (Sokolowski 2010). I believe it is possible to further extend these bee-fly comparisons using the vast genetic and molecular techniques available for *Drosophila* with *Apis* social cues. Fly tools currently unavailable for *Apis*, like GAL4-UAS, can allow for targeted control of gene expression (Duffy 2002). This is especially useful to alter the expression of genes that are involved in conserved pathways of interest in *Apis*. Further, *Drosophila* has

a massive collection of RNAi and mutant lines available to help delve further into understanding the direct functions of specific genes of interest (Dietzl et al. 2007; Johnston 2002). In my analysis, I extended the findings from the above studies and further develop *Drosophila* as a pre-social model in socio-genetic research.

In-line with earlier work, and consistent with the evolutionary idea that social traits are ultimately derived from pre-social Bauplans (Rehan and Toth 2015), I expect the *Drosophila* response to mimic that of bees. Specifically, I predict that male *Drosophila*, as has been seen in females, will behave in a phenotypically similar manner as drones when exposed to the queen bee pheromonal cue. Further, I predict that flies perceive queen mandibular pheromone through olfaction and use olfactory receptor neurons -49b, -56a and -98a to mediate any olfactory response. To this end, I adopted a comparative approach to monitor the behavioural and neural response of specific strains of *Drosophila* to honey bee queen mandibular pheromone.

In this thesis, I test two predictions from sociogenomic theory. First, I used a behavioural assay to test the response of male flies to honey bee social signalling, and compare the observed results to what is expected under a conserved regulatory mechanism – drones display attraction and increase mating effort in the presence of QMP (Chapter 2). Second, I utilized a neural imaging system to map the olfactory neuronal response of flies to the honey bee signal, and identify the specific neurons

involved (Chapter 3). This latter study is a guide to future discovery of these olfactory neurons within the honey bee itself and potentially other social taxa.

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Chapter 2

2 Sexual response of male *Drosophila* to honey bee queen mandibular pheromone: implications for genetic studies of social insects

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2.1 Introduction

The essence of a eusocial breeding system is a reproductive division of labour, in which one or more individuals monopolize reproduction while the remaining majority act as reproductively altruistic helpers (Crespi and Yanega 1995). This reproductive coordination is predicted from inclusive fitness theory (reviewed in Bourke 2011; Marshall 2015), but the precise mechanisms governing social task specialization varies among species (Beshers and Fewell 2001). The European honeybee *Apis mellifera* maintains its division of labour through queen and brood pheromones that signal queen fecundity to her worker daughters (Le Conte and Hefetz 2008; Kocher and Grozinger 2011). Queen mandibular pheromone (QMP) is likely an evolutionarily honest signal (Gadagkar 1997; Keller and Nonacs 1993; Oi et al. 2015; Peso et al. 2015) to which the workers have been indirectly selected to

respond by shutting off their ovaries and otherwise adopting reproductively altruistic roles within the colony (Butler and Fairey 1963; Hoover et al. 2003; Backx et al. 2012).

While this sterility-inducing function of queen pheromone is well understood (Mullen and Thompson 2015), it has a broader role within the honeybee society, and the function of QMP can vary with social context. For example, QMP can induce worker retinue formation, inhibit queen rearing and swarming, and excite congregating males (drones) to mate with virgin queens in flight (Slessor et al. 2005; Free 1987). In this later context, QMP is a mating attractant to which the males orient, and from which they anticipate sex (Gary 1962). As a consequence, a QMP-emitting queen may receive sperm from more than a dozen drones on a single mating flight (Tarpy and Nielsen 2002). From these observations, it is clear that *Apis mellifera* queen mandibular pheromone can coordinate more than one aspect of reproductive behaviour (Brockmann et al. 1998). Like other hymenopteran queen pheromones (Van Oystaeyen et al. 2014), QMP may have evolved through modification of fertility signals already present in pre-social ancestors (Chapuisat 2014; Peso et al. 2015; Oliveira et al. 2015). Unlike other Hymenoptera studied thus far, however, *Apis mellifera* queen mandibular pheromone appears to be relatively derived in its blended chemical composition of volatile carboxylic acids (9-ODA, 9-HDA), aromatics (HOB, HVA) and other compounds, as opposed to a composition of

structurally simple linear or branched alkanes typical of ancestral ants, bees and wasps (reviewed in Oi et al. 2015).

Despite the apparent complexity of QMP there is emerging evidence that the taxon-specific qualities of this pheromone are nonetheless effective at suppressing reproduction via ovary de-activation in un-related insects. Camiletti et al. (2013) showed, for the first time, that female fruit flies (Diptera) exposed to a synthetic blend of QMP respond in a manner comparable to queenright worker bees. That is, they develop smaller ovaries that contain fewer mature eggs, as if they were partially sterile. Moreover, this response to ovary-inhibiting pheromone has a genetic component: *Drosophila* sitter flies (*for*^{S}) are more responsive to the ovary-inhibiting pheromone than are rovers (*for*^{R}), suggesting that the *foraging* gene may in some way mediate this response (Camiletti et al. 2014). These findings are intriguing in that they suggest an underlying pheromone-responsive mechanism that is genetically variable yet sufficiently preserved in flies and potentially other invertebrates (Nayer 1963; Carlisle and Butler 1956; Sannasi 1969). There is, therefore, potential to exploit the *Drosophila* model to uncover conserved genes, pathways and neural circuits involved in ovary-suppression and potentially other fertility-linked traits (Camiletti and Thompson 2016).

Motivated by the prospect of harnessing *Drosophila* gene-finding tools toward questions relevant to honeybee behaviour and biology, I seek to test the effect of

synthetic QMP on male *Drosophila*. I postulate that males might show drone-like behaviour towards QMP. Specifically, I tested if male *Drosophila* respond to biologically-relevant doses of QMP by orienting themselves towards a source of queen bee pheromone – i.e., 'the queen', or if males anticipate sex by increased mating effort towards a conspecific female, or both. If so, I infer that the fertility signal inherent within *Apis mellifera* queen mandibular pheromone and the ability to perceive it is evolutionarily conserved between species from at least one social and one pre-social insect order. I tested both predictions using established *Drosophila* choice and courtship assays, and did so using two control lines and one olfactory mutant genotype that I expect to differ in their responsiveness to olfactory cues.

2.2 Methods

2.2.1 Fly rearing and pheromone treatment

I reared all strains of *Drosophila melanogaster* under standard conditions (25°C, 60% humidity and a 12 h light: dark cycle) in an insect growth chamber (Caron Inc., Marietta, OH) on a standard cornmeal diet as described in Camiletti et al. (2013). I used the *Oregon-R* (*Ore-R*, Bloomington Stock Center #2376) strain of flies as control, for which females have been shown to respond to QMP in a worker-like manner (Camiletti et al. 2016). In addition, I included the $Orco^{1}$ mutant strain in my trials, outcrossed 6 times in w^{1118}. $Orco^{1}$ is homozygous for loss-of-function alleles at the major olfactory co-factor *Orco* locus (formerly Or83b; Larsson et al. 2004) and is

accordingly non-responsive to a wide range of olfactory stimuli (Steck et al. 2012). For all genotypes – that is, *Ore-R, Orco*^{1} and its genetic background control *w*^{1118} – I synchronized adult emergence by housing (for 24 hrs) a small reproductive population (n = 30 males and n = 30 females) in collection cages (60 mm; Diamed, Mississauga, Canada) fitted with nutrient (grape juice and agar) plates. I then collected and transferred day-old larvae to fresh food vials (28.5 x 95mm, VWR International, Radnar, PA) at a density of n = 30 larvae per vial. Finally, I collected same-age, same-sex adult virgins at eclosion (within 1 h) and reared males for three days until sexually mature. To prepare filter papers prior to treatment, I first warmed (50°C in water bath; to render it liquid from a soft wax) and diluted a 500 mg stock of synthetic QMP (Contech Ltd, Victoria, Canada) with absolute ethanol into working aliquots of ~ 13 Qeq units. For reference, 1 Qeq is roughly the amount produced by a single living honey bee queen in 24 h. With bioaccumulation, the pheromone can persist in wax, resin and cuticles within a colony at multi-queen equivalent doses (Hoover et al. 2003; Naumann et al. 1991). I loaded one full aliquot in a final volume of 20 µl onto individual pieces (200 mm^2) of filter paper (grade 413; VWR International, Radnar PA) that I air dried for five hours to ensure ethanol was fully evaporated. For controls, I simply added the equivalent volume of ethanol (no QMP) to filter paper. Each trial was conducted between 12:00 PM – 2:00 PM across multiple days at 23-25 °C, with even light and humidity above 30%.

2.2.2 Testing male attraction to queen pheromone in a T-maze assay

To test if males are attracted to queen pheromone I used a T-maze apparatus (as described in Fernandez et al. 2014). Here, I allowed groups (n = 30) of males to acclimate on the bench top (2 hrs) before transferring them to the maze chamber. The chamber contains left and right 10 cm vials covered with treated or untreated filter paper that is enclosed within endcaps. After five minutes I scored their resting distribution – first, as a proportion of individuals that had oriented to the left or right sides of the chamber, calculated as a preference index (PI; Min et al. 2013; Tully and Quinn 1985), and second, as a virtual frequency histogram of individual flies within the graded (± 1 cm) chamber, as recorded by digital photographs. In each case, I compared the observed distribution of male flies against that expected under a null 50:50 (*i.e.*, no preference) scenario. I replicated this assay for six independent groups of males (from different breeding stocks), and alternated the left and right position of QMP treatment with each trial.

2.2.3 Testing sex attraction under queen pheromone in a courtship arena

Tom tested if males adjust their courtship of conspecific females upon exposure to queen pheromone. Tom scored the courting intensity of individual males using Manning's (1960) scheme, which reflects the stereotypical escalation of courtship intensity towards females: 1- orientation, 2- tapping, 3- singing, 4- licking, 5-

attempted copulation, and 6- successful copulation. In instances where a male did not attend to the female, Tom assigned a score of zero. Here, Tom reared *Ore-R* flies as above, except to specifically motivate males towards mating, Tom housed them from eclosion as individuals in separate vials. Virgin females were also collected at eclosion but all were housed together in single vials. After three days, or as courtship is expected to peak (Kosuda 1985), Tom transferred one CO_2-anaesthetized male and female into a Lab-Tek™ tissue culture chamber (20 x 10 mm; Division Miles Laboratories Inc., Naperville) with filter paper that served as a courting arena. After a 10 min habituation period in the chamber, Tom video tracked courting behaviour for a continuous 10 mins using Zeiss Zen Pro stereomicroscope software (Carl Zeiss Canada Ltd, Toronto), and did so for n = 22 QMP-treated and n = 22 control trials.

From video playback, Tom recorded start and stop times (to the nearest 1s) of each behavioural event. This combination of information on frequency and duration of specific male behaviours allowed me to estimate four different measures of mating effort. First, Tom estimated *courtship latency* – the time to initiate courtship (O'Dell 2003). In this case, Tom scored the time to reach a score of '1' (orientation) for the first time. Second, Tom estimated *overall mating effort* – as measured by the graphical area under a time-by-intensity profile curve (Equation B.1). Next, Tom estimated the *courtship index* – simply, the proportion of time spent in courtship (Siegel and Hall 1979). Finally, Tom estimated the *low courtship intensity* – the time

a male spends courting within the lower tier of intensity scores (1-2), and the *high courtship intensity* – the time a male spends courting within the upper tier of intensity scores (4-6). For each of the five measures, both Tom and I tested for differences between treated and control male courtship effort using unpaired one-tailed *t*-tests, and applying a Benjamini-Hochberg correction for multiple (*i.e.*, 5) testing (*q*-value; Waite and Campbell 2006).

2.3 Results

There is a strong effect of queen bee pheromone on the choice made by male *Drosophila*, as evidenced by their distribution within the T-maze. I found a genotype-dependent response to pheromone is captured in a significant gene-by-treatment interaction effect (from a two-way ANOVA; $F_{2, 30} = 6.47$, $P = 0.005$). *Ore-R* flies consistently orient toward the pheromone, as indicated by a positive PI relative to untreated *Ore-R* controls (mean PI = 0.361 vs. 0.022; $t = 5.40$, $P < 0.001$; Figure 2.1). This effect of pheromone on fly behaviour is dependent on fly genotype; $Orco^{1}$ mutants show no preference for QMP and their PI is, therefore, not different from zero (one-sample *t*-test; mean PI = -0.016; $t = 0.284$, $P = 0.783$). Finally, white-eyed controls behave as *Ore-R* flies, and are likewise biased in their distribution towards the source of pheromone (mean PI = 0.334 vs. 0.039; $t = 3.646$, $P = 0.004$). My effort to capture the living distribution of individual flies yields results that are consistent with the proportional calculation reflected in the PI; *Ore-R* males orient

towards the pheromone, regardless of the actual polarity (left vs. right) of the apparatus (*z*-score = -2.94, *P* = 0.003; Supplementary Figure B.1). Conversely, *Orco*^{1} mutants do not deviate from a uniform distribution (*z*-score = 0.168, *P* = 0.860) and therefore appear indifferent to the pheromone.

There is a subtle but significant effect of queen pheromone on fly courting behaviour. From video-capture trails, Tom reconstructed the behavioural courtship profile of each male in the form of a score-by-time ethograph (Figure 2.2a). First, the number of males showing non-zero courting scores was not statistically different between QMP-treated and control trails (n = 19 vs. 17; χ^2 = 0.61, *P* = 0.43). Of the males that did court (had a score 1-6), exposure to queen pheromone did not significantly affect the mean time to initiate courtship (*courtship latency*, *t* = 1.44, d.f. = 34, *q* = 0.126; Figure 2.2b), but did increase the overall mating effort (area under a time-by-intensity profile curve, *t* = 2.25, d.f. = 34, *q* = 0.040; Figure 2.2c). Further, exposure to pheromone did not increase the overall proportion of time spent in courtship (*courtship index*, *t* = 1.00, d.f. = 34, *q* = 0.162; Figure 2.2d), nor did it affect the proportion of time spent by males orienting towards and tapping females (*low intensity score, t* = 1.30, d.f. = 34, *q* = 0.126; Figure 2.2e). Pheromone-exposed males did, however, spend a greater proportion of time licking, attempting to copulate, and copulating with females (*high intensity score*, *t* = 2.29, d.f. = 34, *q* = 0.040; Figure 2.2f), relative to untreated males.

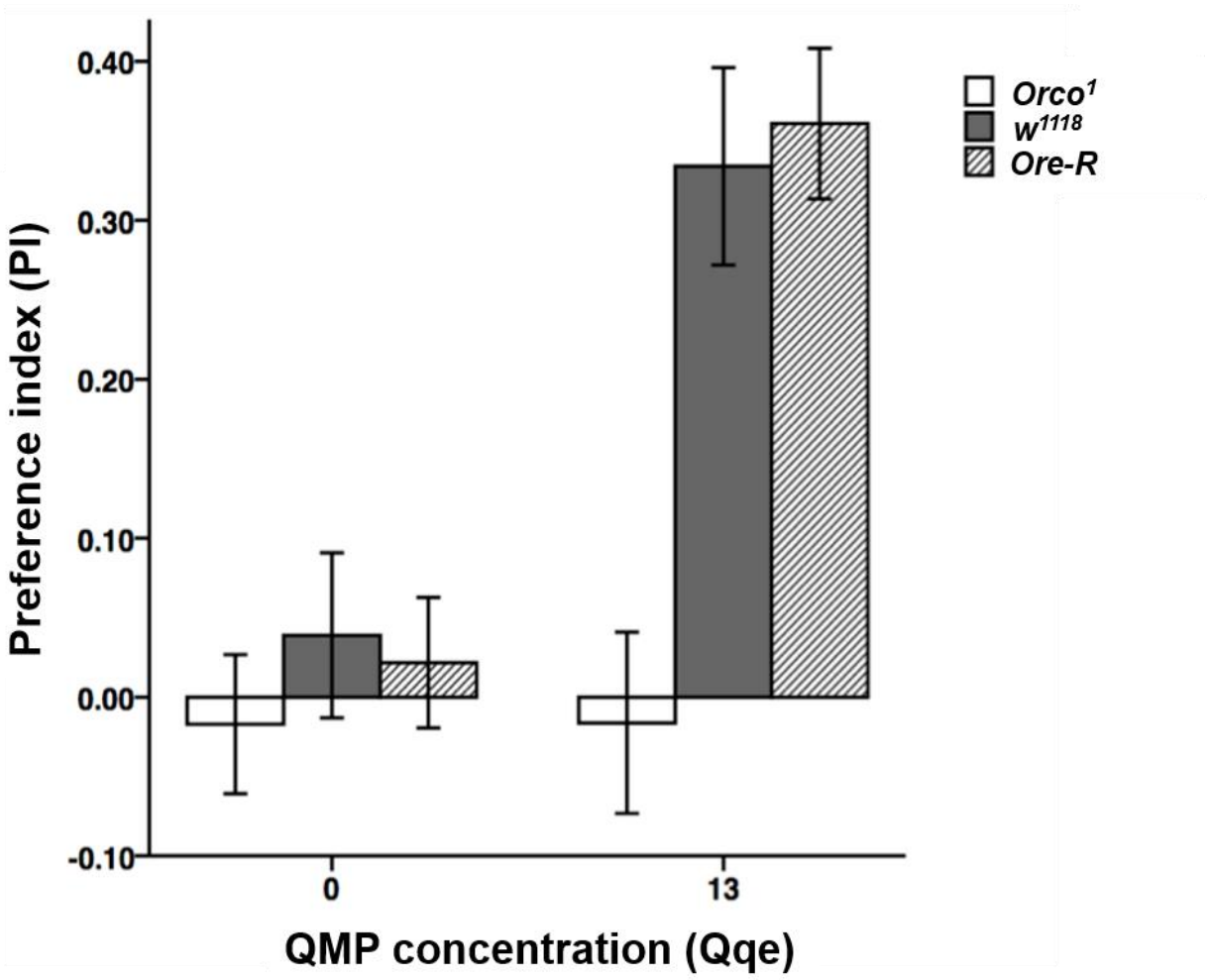

Figure 2.1 Mean preference index for male flies as a function of genotype and pheromone treatment. A 50:50 distribution (no preference) would yield a PI of zero, and a fully biased distribution towards QMP+ would yield a PI of 1. Both the *w*^{1118} and *Oregon R* genotypes show significant preference for queen pheromone, whereas *Orco*^{1} mutants, and all untreated flies, showed no preference. Error bars show ± SEM.

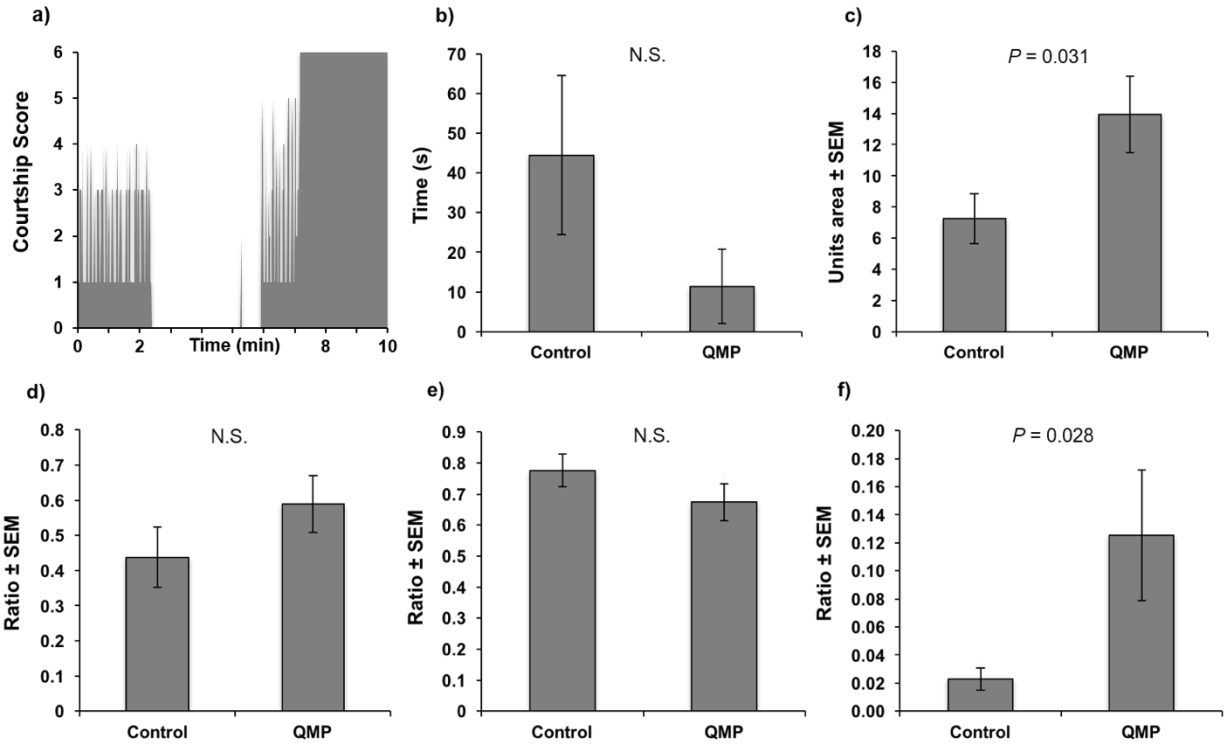

Figure 2.2a Typical male courtship profile showing his courtship score (*y*-axis) for each second of the observation period (*x*-axis). From each focal individual's profile, Tom calculated mating effort in five unique but comparable ways: **b** *Courtship latency* - the time to initiate courtship (see text for full definitions), **c** *Overall mating effort* - the area under each individual male's behavioural courting profile curve, **d** *Courtship index* – fraction of time courting (1-6) in relation to total time of trial, **e** *Low courtship intensity* – time spent in lower tier (1-2) of courtship intensity scores, and **f** *High courtship intensity* – time spent in higher tier (4-6) of courtship intensity score. The uncorrected *P*-values shown for **c** and **f** remain significant when adjusted for multiple testing (*q*-value < 0.05 in each case). Otherwise, N.S. = non-significant.

2.4 Discussion

According to social signalling theory, the queen pheromones that coordinate reproductive roles within honey bee and other social hymenopteran societies may be derived from fertility signals of pre-social taxa (Chapuisat 2014; Peso et al. 2015; Oliveira et al. 2015; Oi et al. 2015). My results provide two observations consistent with this idea. First, I show that *Drosophila melanogaster* males are attracted to honeybee queen mandibular pheromone. This is evident by orientation of *Ore*-R males toward the source of pheromone within a T-maze chamber. This behaviour pattern suggests that male *Drosophila*, despite their phylogenetic position and pre-social biology, can respond to a eusocial honey bee fertility signal. I can inhibit this response through a loss-of-olfactory-function mutation, which suggests the behavioural response is mediated by olfaction. Second, exposure of male flies to queen pheromone increases aspects of their reproductive effort towards conspecific females. Pheromone-exposed males spent more time courting females at 'high intensity' (licking, attempting to copulate, and copulating), but QMP did not otherwise obviously affect male fly behavior. These two conspicuous behavioral responses from *Drosophila melanogaster* – attraction and mating intensity – to a bee pheromone is potentially significant because it mimics the fertility signal's normal effect on male bees. Like drones (Slessor et al. 2005; Free 1987; Brockmann et al. 2006; Butler and Fairey 1964), male flies are attracted to, and display increased intensity to court with, pheromone-emitting queens. This basic comparison between

fly and bee behavior to a single pheromone suggests a conserved mechanism for sexual signaling between social and pre-social insect orders.

The preference index is widely applied to T-maze behavioral assays (Min et al. 2013; Suh et al. 2004; Tully and Quinn 1985) and provides a normalized measure of variance in dispersion. In this case, *Ore*-R flies showed a range in PI-value from near zero (untreated control populations) to a PI-value of ~0.35 (pheromone treated population). This range is consistent with comparable studies (e.g., Min et al. 2013) that score the behavior of small (~20-50 individuals) populations of *D. melanogaster* and indicates a moderate level of attraction that is well above baseline. This attraction to pheromone is, however, a function of genotype, as olfactory deficient mutants showed unbiased PI-values. This null result for $Orco^{1}$ mutants is consistent with my no-pheromone controls (blank filter paper vs. evaporated ethanol; not shown) and with my prediction that flies strongly deficient in function for olfactory sensory neurons would not likely detect the pheromone. Given that *Orco*, by any name (Vosshall and Hansson 2011), plays a conserved and essential role in insect olfaction (Krieger et al. 2003; Larsson et al. 2004), I reason that the *Drosophila* response to QMP is at least partly dependent on olfaction. Here I follow precedent (Camiletti et al. 2013; Camiletti et al. 2016; Camiletti et al. 2014) and use a naturalistic blend of synthetic pheromone to induce changes to fly reproductive phenotype. Using a multi-component blend that mimics the natural bee pheromone presumably has the best potential to capture additive and synergistic effects among

the individual pheromone components, as it does in bees (Hoover et al. 2003; Slessor et al. 2005; Brockmann et al. 2006). I have not yet tested single components of QMP on *Drosophila*, but I predict that 9-ODA ((2E)-9-oxodecenoic acid) may elicit the strongest single-component attraction and aphrodisiacal effect on male flies, as it appears to in *Apis mellifera* drones (Brockmann et al. 2006; Free 1987).

I here suggest that males, as for females (Camiletti et al. 2016), perceive the pheromone through olfactory neurons, but I do not yet know which neurons are specifically excited by this or related pheromones, or whether neurons are functionally segregated between males and females. Current work suggests that neurons associated with olfactory receptors Or-49b, Or-56a and Or-98a may specifically be necessary for female detection of QMP (Camiletti et al. 2016). If this response is dependent on olfactory neural circuitry then it may be possible to exploit a neural map of *Drosophila* (Chiang et al. 2011) and *in vivo* calcium imaging (Masuyama et al. 2012) to infer a detailed connectome associated with the fly's response to QMP. A first-generation neural map of the aphrodisiac (male) or anti-ovarian (female) response to queen bee pheromone, even from *Drosophila*, could complement the emerging framework for understanding how honey bees and other social animals modulate social information to regulate personal reproduction (Robinson et al. 2008; Sokolowski 2010; Hofmann et al. 2014; Thompson and Richards 2016).

Scoring *Drosophila* courtship behavior is well established, and includes schemes that rank courtship progression and intensity along an ordinal scale (Hall 1994; Ruedi and Hughes 2008). I predicted that queen bee pheromone might induce a comparable response from male flies as it does from drones – namely to increase motivation for sex. I found that male *Drosophila* can augment aspects of their mating effort, but my evidence for an aphrodisiac effect was subtle. Specifically, I found that pheromone-exposed males tended to prolong the more 'intense' aspects of their courting effort towards females – that is, they tend to spend longer courting at higher intensities (*high intensity mating score*) than simply courting for longer (*courtship index*). This subtle yet biologically relevant time-by-intensity effect of pheromone on *Drosophila* courting behaviour would not be apparent if I had used only standard mating progression metrics.

Taken together, the behavioral response of male flies to bee pheromone that I observe in this study is striking for two reasons. First, *Drosophila* would never normally be exposed to a bee pheromone, and thus their conspicuous response is unlikely to be adaptive, at least not specifically adaptive to *Apis mellifera* QMP. Instead, the response suggests a conserved mechanism that mediates perception of information that is functionally conserved between species from distantly related eusocial (Hymenoptera) and pre-social (Diptera) orders. Of course it should not be overstated that flies could be perceiving QMP as food (HOB is found naturally in some plants like *Oxalis tuberosa*; Pal Bais et al. 2003) and thus this could be

mediating their response. Second, social signaling theory suggests that even complex social traits ranging from group nesting, foraging and defense, to stark divisions in reproductive labour likely evolved as socially-modified versions of simple, non-social behaviours (Reaume and Sokolowski 2011). If so, then *Drosophila* may provide a genetically tractable model (Hales et al. 2015) to test for conserved genetic, gene regulatory and neural effects on some social traits (Camiletti and Thompson 2016).

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Chapter 3

3 Mapping neurons that respond to social pheromone: insights from a *Drosophila* model

3.1 Introduction

The division of labour typical of eusocial species likely evolved via disruptive selection on reproductive versus non-reproductive phases of a pre-social lifecycle (Hunt and Amdam 2005). If so, I can expect some homology between reproductive cycles of pre-social insects, in which these phases remain as environmentally-responsive options to a single individual, and eusocial castes, in which 'phases' have become more-or-less fixed amongst individuals (Rehan and Toth 2015). One expectation from this 'reproductive ground plan' hypothesis (West-Eberhard 1996; Graham et al. 2011) is that pre-social and eusocial species may respond similarly to environmental cues that regulate reproduction, provided their sensory and reproductive systems remain sufficiently conserved (Camiletti and Thompson 2016). The idea here is that the co-regulated gene sets of pre-social ancestors have been altered during social evolution to direct social divisions in labour (Johnson and Linksvayer 2010).

To predict that an individual eusocial insect might regulate personal reproduction in a manner comparable to, say, *Drosophila*, or some other non-social insect seems unlikely, especially given their distant ancestry. However, female honey bees do de-

active their ovaries in response to nutritional, thermal or other forms of stress, as do many insects (Paul and Keshan 2016; Xu et al. 2009; Cui et al. 2008), including *Drosophila* (Neckameyer and Weinstein 2005). This widespread pattern suggests that there are some basic features of reproductive regulation that are widely conserved among insects. Likewise, female *Drosophila* can de-active their ovaries in response to social cues derived from eusocial bees (Sannasi 1969), and do so in a manner that is comparable to a (female) worker bee's normal response to ovary-inhibiting queen pheromone (Camiletti and Thompson 2016). Given the apparent homology in systems governing reproductive regulation, even between these two phylogenetically distant (Diptera vs. Hymenoptera) insects, it may be possible to exploit the response from *Drosophila* to queen pheromone, and begin to identify the earliest-acting neural receptors that initiate the ovarian response in flies.

Camiletti et al. (2013) showed that application of honey bee queen mandibular pheromone (QMP) to virgin female flies can induce a worker-like response in the form of fewer mature eggs and smaller ovaries, relative to untreated controls. This inter-specific response from a pre-social insect to a eusocial pheromone has since been observed in male *Drosophila* that are positively attracted to queen pheromone and, like drones, increase their interest in courting conspecific females (Chapter 2). To the extent that *Drosophila's* response to QMP is comparable to that of *Apis*, I can consider the fly an unconventional but nonetheless genetically tractable model for testing certain hypotheses relevant to insect sociobiology.

For example, Camiletti et al. (2016) used an RNAi knock-down screen to identify several candidate olfactory receptors (Or) that may regulate the observed response from flies to QMP. The study implicates Or-49b, Or-56a and Or-98a as being responsive to queen pheromone. Each of the three of these receptors map to the same tissue type (basiconic sensilla) on the antenna (Figure 3.1). I therefore predict that exposure to QMP will stimulate these three neurons. I further predict that olfactory receptors that were not implicated by Camiletti et al. (2016) as being queen bee pheromone responsive will not be stimulated by QMP.

In order to test these predictions, I used a nuclear factor of activated T-cell (NFAT) system to identify specific olfactory neurons that are stimulated by honey bee queen mandibular pheromone. Within this system sustained neural activity initiates calcium accumulation. Calcium activates calcineurin that dephosphorylates NFAT, causing the chimeric transcription factor mLexA-VP16-NFAT to shuttle into the nucleus. Once inside the nucleus, the chimeric transcription factor induces expression of the GFP reporter gene, which is under the control of the LexA operator (LexAop; Masuyama et al. 2012). Further, this system also takes advantage of the Gal-4/UAS system which only allows the chimeric transcription factor to be transcribed in specifically chosen tissues (Duffy 2002). This dynamic reporter system has previously been used to label neuronal receptors responsible for the perception of DEET (Kain et al. 2013), as well as fly cuticular chemical extracts (Masuyama et al. 2012), among other ecologically relevant chemical stimuli. Based on this

precedence, I propose that Drosophila's NFAT system is potentially suitable for identifying olfactory receptors responsive to queen pheromone. Here I report my use of the NFAT system to test whether Drosophila Or-19a, -49b, -56a, -85a and -98a are responsive to QMP.

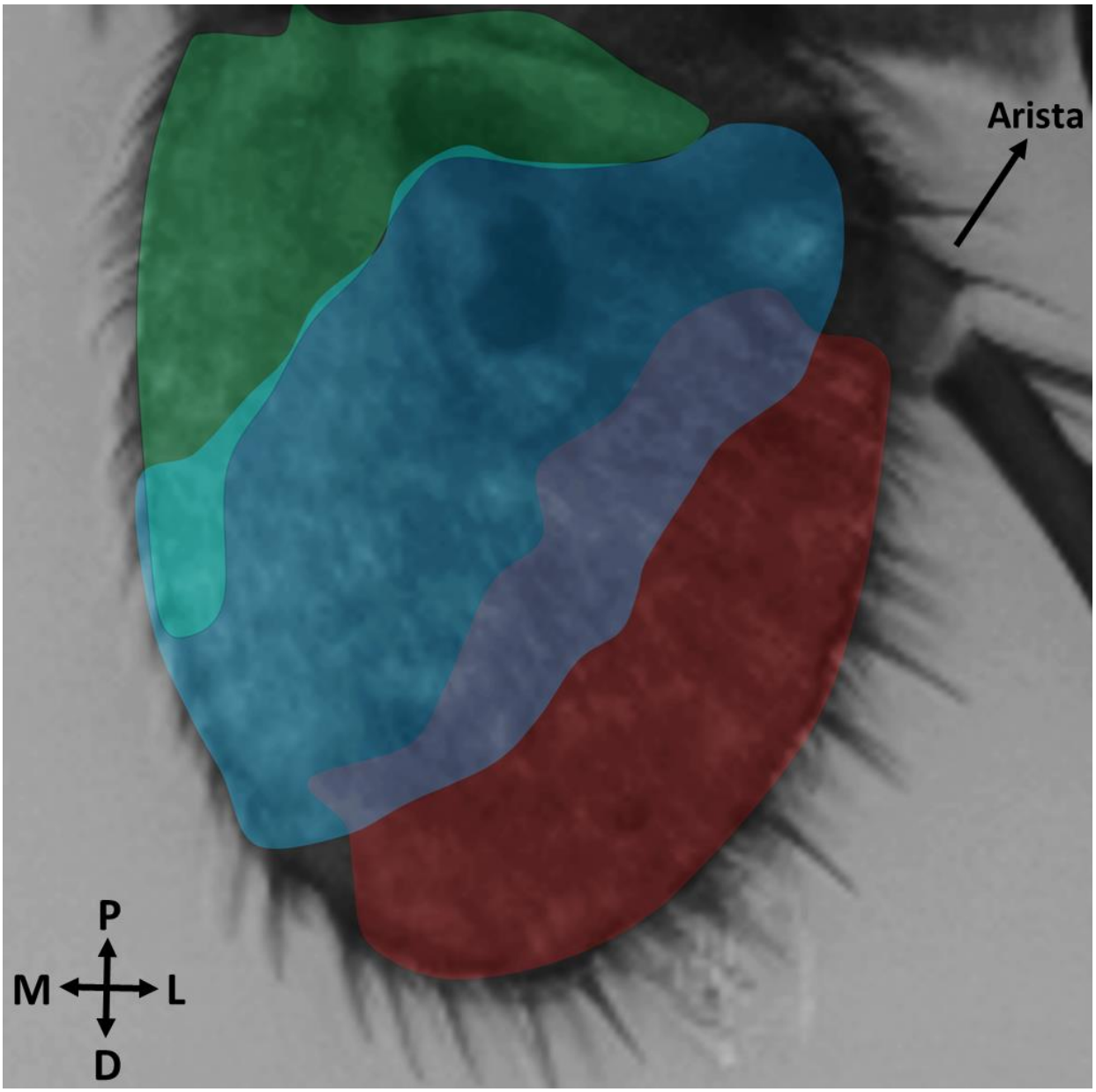

Figure 3.1 A reconstructed z-stack confocal micrograph of a fly antenna overlaid with the distribution of the three types of sensilla on a fly antenna (modified from Couto et al. 2005). The three types are: large basionic sensilla (green), the thin and small basionic sensilla (blue), and the trichoid sensilla (red). Abbreviations: P, proximal; D, distal; M, medial; L, lateral

3.2 Methods

3.2.1 Fly rearing

All strains of *Drosophila melanogaster* were reared under standard conditions (25°C, 60% humidity and a 12 h light: dark cycle) on a standard cornmeal diet (as in Camiletti et al. 2013) in an insect growth chamber (Caron Inc., Marietta, OH). Further all lines (Table 3.1) were acquired from the Bloomington Stock Center (Bloomington, IN, USA), with the exception of the NFAT line itself which was donated by Dr. Anthony Percival-Smith (Western University, Canada).

Table 3.1 A summary of the olfactory line genotypes used in the assay. Shown is the olfactory receptor (Or) common name and the corresponding the Bloomington Stock Center ID (BSC ID) from which the transgenic fly was made.

UAS Strain (female)		Gal-4 Strain (male)	
		Common Name	BSC ID
LexAop-CD8-GFP-2A-CD8-GFP; UAS-*mLexA*-VP16-NFAT, *LexAop*-CD2-GFP/+	*Crossed to:*	elav	8765
		Or-19b	23888
		Or-49b	24614
w*; P{10XUAS-mCD8::GFP}attP2 (32184)		Or-56a	9988
		Or-85a	23133
		Or-98a	23142

3.2.2 Pheromone treatment

Prior to treating flies with synthetic queen pheromone (Contech Ltd, Victoria, Canada), I first prepared 13 'queen equivalent' (Qeq) doses in 20 µl volumes of ethanol (as described in Camiletti et al. 2013). I then warmed (50°C in water bath) this volume prior to dispensing it onto filter paper (200 mm^2, grade 413; VWR International, Radnar PA) that served to deliver pheromone to populations of flies. For each trial, I collected virgin male and female flies within two hours of eclosion, then maintained them individually (1 fly per vial) for three days in an odour free environment. At maturity, I exposed small groups (n = 2-3 same sex individuals) of flies for 24 hours (27 °C) to one of two treatments via a custom chamber (see Camiletti et al. 2016) in which flies could not touch or eat the QMP but could smell it. Because ethanol (solvent) itself can potentially affect fly behaviour and physiology (Devineni and Heberlein 2012), I air-dried each filter paper for five hours until the ethanol was evaporated. For no-pheromone controls, I just dispensed the equivalent volume of ethanol onto the filter paper.

3.2.3 Neural imaging

Following exposure, I anaesthetized flies on ice and removed one antenna under a cold Dulbecco's phosphate-buffered saline solution (DPBS). I then fixed (4% PFA, 30 mins), washed (three times in PBS w/ 0.3% Triton X), and mounted (in glycerol solution) antennae on glass slides. I acquired z-stack fluorescent image data from

each slide via confocal microscopy (Zeiss LSM 510 confocal microscope and Zen Light Edition software) and reconstructed the several images into one for analysis. Specifically, I scored the neurons, and regarded the neuron as 'active' if it fluoresced. I also used IMAGEJ software to standardise the size of each antenna and precisely map the area on the antenna that fluoresced, and thus presumably contained the neurons of interest. To control for observer bias, I scored images blind to the treatment or genotype.

To gauge significance in degree of neural activity, I visually compared separate groups based on the following two criteria: 1) neuronal fluorescence was clearly present on the antenna, and 2) the location of fluorescence was the same or different between groups. First, I compared QMP-treated neuronal-driven (8765) NFAT flies and their untreated controls. Next to infer if specific olfactory receptors were activated with the queen pheromone, I compared NFAT flies crossed to specific olfactory receptors (Or-19a, -49b, -56a, -85a, -98a) in treated and untreated groups. Finally, acting as a positive control, I crossed UAS-GFP flies to the same olfactory receptors. This control was visually compared to QMP-treated neuronally driven NFAT flies to assess if the olfactory receptors themselves were located in the same area as QMP was perceived on the antenna.

For any olfactory receptor deemed to be involved in the response of QMP in *Drosophila melanogaster* I ran a BlastP search for protein homologs in *Apis*

mellifera. I only accepted matches with expectation values < 0.001 as these can reliably be used to infer homology (Pearson 2013).

3.3 Results

I found that exposure to QMP showed an increase in expression of GFP in neurons of the *Drosophila* antenna over untreated controls when NFAT was active in all olfactory receptors (Figure 3.2). This fluorescence pattern appears concentrated to an area adjacent to the arista, and across both the large and small basiconic regions. This general fluorescent pattern indicates that my NFAT lines and Gal-4/UAS driving system were working as expected.

The Or lines crossed with UAS-GFP were compared to the observed location of antennal QMP perception Figure 3.2 where QMP was found to be perceived on the antenna. Upon visual inspection I found that olfactory receptors Or-49b (Figure 3.3F), Or-56a (Figure 3.3G), Or-85a (Figure 3.3J) and Or-98a (Figure 3.3H) all fluoresce within the same area QMP was perceived when all olfactory neurons were active (Figure 3.2). One olfactory receptor, Or-19b, fluoresced in a separate region of the antenna that is away from where QMP is sensed on the antenna (Figure 3.3I).

Furthermore, when the calcium dependant NFAT system was driven in the specific receptors, only Or-49b (Figure 3.3K), Or-56a (Figure 3.3L) and Or-98a (Figure 3.3M) displayed antennal fluorescence when exposed to QMP over untreated controls (Figure 3.3A-C). This was not the case for the other two olfactory receptors that I

tested: Or-19b (Figure 3.3N) and Or-85a (Figure 3.3O) did not fluoresce, and were thus not different in this respect to the untreated controls (Figure 3.3D-E).

From this preliminary analysis Or-49b, Or-56a and Or-98a seem to be the most likely candidates as functional QMP receptors in the fly. Using these QMP functional receptors, I found strong levels of statistical homology between known *Apis* protein sequences in GenBank with each of the *Drosophila* olfactory receptors (Table 3.2).

Table 3.2 The *Apis mellifera* homologues retrieved for *Drosophila melanogaster* QMP sensing olfactory receptors using BlastP. Only *E*-values below 0.001 are shown. The lower the *E*-value, the lower probability is of finding a match by chance. Coverage denotes only the percentage of the query that aligns with a hit.

Drosophila OR	*Apis* OR homolog	*E*-value	Coverage
Or-49b	*Am*Or-14	2e-14	64%
	*Am*Or-51	1e-4	58%
	*Am*Or-78	2e-17	80%
	*Am*Or-94	6e-14	85%
	*Am*Or-160	2e-5	60%
Or-56a	*Am*Or-4	2e-8	40%
	*Am*Or-78	4e-4	35%
Or-98a	*Am*Or-160	9e-4	33%

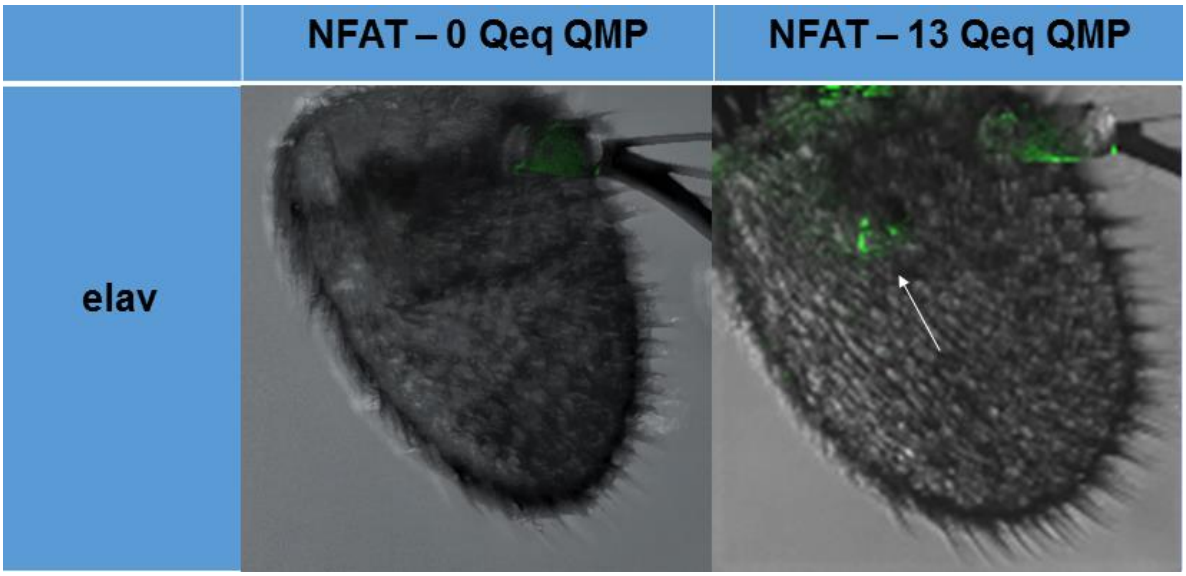

Figure 3.2 Reconstructed z-stack confocal micrographs of antenna from neuronal-driven NFAT *Drosophila* stimulated with 13 Qeq of QMP or ethanol solvent (0 Qeq). The green fluorescent indicates the location of neurons that are activated under exposure to QMP.

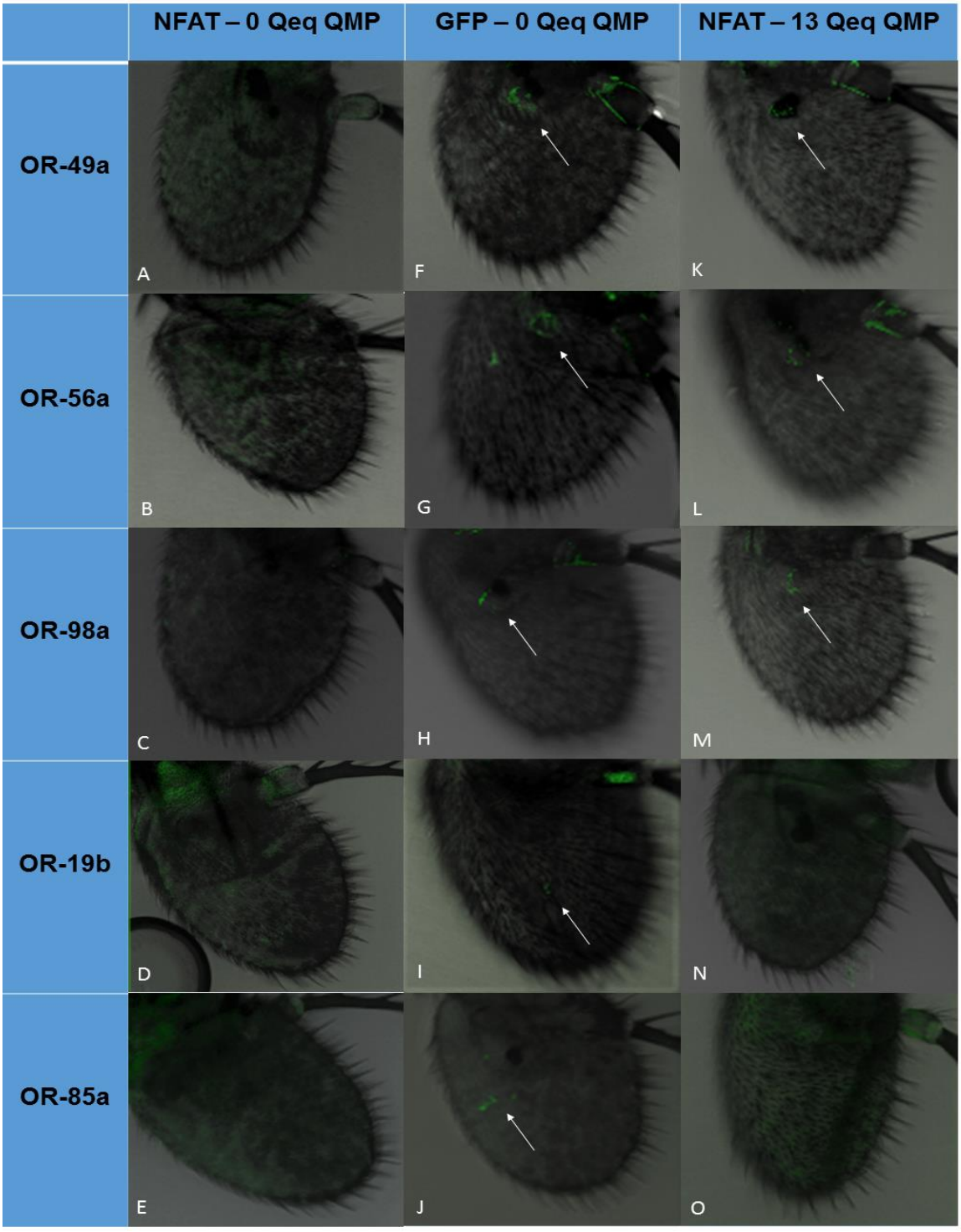

Figure 3.3 Reconstructed z-stack confocal micrographs of antenna from specific olfactory driven NFAT *Drosophila* stimulated with 13 Qeq of QMP or solvent (ethanol). Antenna from specific olfactory driven GFP *Drosophila* act as a positive control. White arrows are used to bring attention to areas of GFP.

3.4 Discussion

In this study, I utilized a calcium-dependant nuclear factor of activated T-cell (NFAT) system to report specific olfactory receptor neurons that are putatively responsive to a bee pheromone. My results are preliminary but implicate three neurons (Or-49b, Or-56a and Or-98a) as functional in the perception of QMP, as evidenced by their specific fluorescence upon exposure to pheromone. Further, my screen rejected two other olfactory receptor neurons (Or-19a and Or-85a) that I included in my screen but did not expect to test positive. My combined result is important for two reasons. First, the three olfactory receptor neurons that I identified can further be suggested as prime candidates for the very neuron homologues used by *Apis* itself to detect queen pheromone. Second, my results continue to promote a potential role for pre-social *Drosophila* as a genetically tractable model for socio-genetic research.

Despite having 350 million years of divergent evolution between them (Kazemian et al. 2014), and structurally very different antenna (Chapman 1998), *Drosophila* and *Apis* may, nonetheless, share some common neural features for the perception of social pheromones. In both insects, signal detection is initiated by olfactory receptor neurons located within sensilla on the antennae (Sato and Touhara 2009). Odorant molecules ligands bind to a structurally compatible receptor, and the signal is carried via the antennal nerve to the antennal lobe, the primary olfactory center within the insect brain (Sato and Touhara 2009). In this study, I found evidence for a functional

role for specific olfactory receptor neurons, which compliments behavioural studies that previously suggested a role for olfaction in *Drosophila*'s perception of QMP (Chapter 2; Camiletti et al. 2013).

I further established, for the first time, the utility of the NFAT system within a sociobiological framework. As such, I identified QMP-sensitive neurons. I based this study of the olfactory receptor neurons by asking two questions. First, did QMP allow for fluorescent neurons to be observed anywhere across the antenna? And, second, were they observed in the precise region where neuron-active flies displayed QMP perception? My analysis demonstrated that queen bee pheromone interacts with olfactory receptors adjacent from the arista and located medially on the antenna. I expected that Or-49b, Or-56a, Or-85a and Or-98a were all located in this region (Couto et al. 2005), and my assay confirmed this prediction. Further, only Or-49b, Or-56a and Or-98a were active with QMP, and therefore, my results generally support the RNAi-screen of Camiletti et al. (2016) that first implicated these receptors in the fly.

All of the QMP responsive neuronal olfactory receptors found in *Drosophila* were expressed in the thin and small basiconic sensilla. The olfactory receptor neurons found within these sensilla respond strongly to food odors such as fruit (de Bruyne et al. 2001; Hallem et al. 2004) and mold (Stensmyr et al. 2012). While the individual receptors have not been fully characterised, Or-49b is known to be responsive to

aromatics and Or-98a is responsive to terpenes, ketones, aromatics, alcohols and esters (Hallem and Carlson 2004). Furthermore, Or-98a seems to share a link with regulation of sexual receptivity (Sakurai et al. 2013).

Gaining insight into the precise olfactory receptor neurons that flies use with a social pheromone is potentially significant if it helps to identify these neurons in the honey bee itself, or other social insects in which reproductive conflict and cooperation is mediated through pheromones. To date only one odorant receptor for QMP perception in honey bees has been established. *Am*Or-11 responds specifically to 9-ODA, the most abundant portion of QMP (Wanner et al. 2007). To guide future *Apis* studies, I did a direct BLASTP of each of the three QMP responsive neuronal receptors against the *Apis mellifera* genome (Amel_4.5). While the coverage varied greatly (33 - 85%) in related honey bee olfactory receptors, I only accepted those protein sequences with low enough *E*-values (<0.001) to suggest homology (Pearson 2013). I found that *Apis* olfactory receptors *Am*Or-4, -14, -51, -78, -94 and -160 show homology to the *Drosophila* olfactory receptors implicated with queen bee pheromone response. These predicted *AmOr* receptors, while different from the Camiletti et al. (2016) predicted bee Or's using genealogical relationship and olfactory receptor ligands similarity to components of queen pheromone, suggests these receptors can be used as candidates for future functional testing within the bee itself.

I expect that these olfactory receptors, when knocked-down, could alter various behavioural and physiological traits related to honey bee social functions. Further, since both workers and drones do respond to, +/- 9-HDA, HOB and HVA, the other four major components of QMP (Free 1987), this assay could be expanded to further look at the individual components of QMP and understand precisely which olfactory receptors flies use to respond to each of these components.

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Chapter 4

4 Concluding remarks

4.1 General discussion

Evolutionary developmental biology (Evo-Devo) often adopts a comparative approach, and thereby, uses phylogenetic information to test how phenotypes have diverged or converged over evolutionary time (Laubichler 2007; Carroll et al. 2005; Raff 1996). Just as this field has revealed genes that are repeatedly co-opted across the tree of life to solve functionally similar problems - e.g. the *pax-6* gene for eye development (Gehring and Ikeo 1999) - the field of sociobiology may also benefit from a comparative approach (Toth and Robinson 2007; West-Eberhard 2003; Kapheim et al. 2015). Specifically, we can expect that some of the genes involved in reproductive regulation - that is, involved in mediating trade-offs between present and future reproduction - in social taxa will be homologous to the genes that coordinate similar trade-offs in pre-social and even non-social taxa. The so-called ovarian ground plan hypothesis (West-Eberhard 1996; Graham et al. 2011; Rueppell et al. 2008) captures this idea. It suggests that the reproductive division of labour that is obvious between queen and worker castes of social Hymenoptera (Beekman and Ratnieks 2003) evolved through disruptive selection on gene networks that once coordinated individual reproduction of solitary female insects (Amdam et al. 2006;

West-Eberhard 1996). For example, vitellogenin (Nelson et al. 2007) and insulin (Wang et al. 2009) signaling pathways that once regulated transitions between the reproductive and non-reproductive phases in pre-social life cycles now appear to regulate the differentiation of reproductive and non-reproductive castes. This apparent co-option of ancestral pathways from solitary life histories into socially-coordinated reproduction suggests that there may be widespread homology in the mechanisms that regulate reproduction in social and non-social taxa.

While we do expect some genes and pathways to be conserved between pre-social and eusocial taxa, we can also predict a role for new genes and other forms of genetic novelty in social evolution (Sumner 2014), and the extent of 'old' versus 'new' genes likely varies among social systems and taxa (Johnson and Linksvayer 2010). Old genes and pathways can also be regulated in new and novel ways that give rise to social traits (Robinson and Ben-Shahar 2002; Berens et al. 2015; Mikheyev and Linksvayer 2015). One way to test these ideas is to compare social against pre-social or non-social taxa for genes and pathways that may function similarly. Carpenter bees (Rehan et al. 2014) and paper wasps (West-Eberhard 1996) have relatively simply social systems that may help to model the early stages of eusocial evolution. For example, comparing behavioural differences in nesting biology of a solitary bee species, the sweat bee (Halictidae; Boesi et al. 2009), with that of the eusocial honey bee provided insight into the intermediate steps in evolution of from solitary to eusocial behaviour.

A comparative approach might also be extended to include pre-social species outside of Hymenoptera. Recent studies have suggested a role for *Drosophila melanogaster* as a model in social insect biology. *Drosophila* is an established model taxon in evolutionary and behavioural genetics (Powell 1997). This genus of fly has some social attributes - for example, it is gregarious in that females can share nesting sites (Parsons and Stanley 1981) - but *Drosophila* lacks cooperative brood care or division of reproductive labour, so they cannot be considered social *per se* (Gadagkar 1987). Despite the potential for some species of *Drosophila* to inform theories on pre-social biology, it has not been widely used in a sociobiological context (Camiletti and Thompson 2016; Hasselmann 2015). In this thesis, I present the view that this model may serve as a useful - if not crude - proxy for testing specific ideas on the genes, neurons and behaviours used to regulate reproduction within a social environment. Though the reproductive and social biology of fruit flies is not obviously comparable to that of social Hymenoptera it may, nonetheless, prove to be useful given its pre-social biology, and wide array of behavioral genetic tools that are available (Camiletti and Thompson 2016). Using a *Drosophila* model, may, therefore, make it possible to test the behavioural, genetic, and neural homologies between pre-social and eusocial taxa.

In general, the benefits of using *Drosophila* as a model include their ease of laboratory manipulation, their fully sequenced and annotated genome, and the many tools available for genetic manipulation (Lin et al. 2014). These well-developed

genetic tools – like GAL4-UAS (Duffy 2002) and RNAi (Boutros et al. 2004) - have allowed for in-depth studies of specific genes in well-designed assays made specifically for *Drosophila*, including the T-maze (Tully and Quinn 1985) and courtship assays (Manning 1960). Outside of studying behaviours, *Drosophila* can be further manipulated to observe the neuronal and sensory systems that underlies their response to various stimuli. Both electrophysiology (de Bruyne et al. 2001) and the nuclear factor of activated t-cells system (NFAT; Masuyama et al. 2012) have been used to pinpoint specific neuronal responses. Utilizing these types of techniques for pre-social *Drosophila* within a social context could assistance in identifying the conserved genes and multi-gene networks from which eusociality evolved.

The goal of my thesis was to use *Drosophila melanogaster* as a behavioural and neuronal model to test how conserved is the mechanism through which one species of honey bee (*Apis mellifera*) and one species of fly regulate their reproductive behaviour. In Chapter 2 - 'Sexual response of male *Drosophila* to honey bee queen mandibular pheromone: implications for genetic studies of social insects' - I show that male fruit flies respond to honey bee queen mandibular pheromone in a manner that suggests behavioural homology to drone bees. In Chapter 3 - 'Mapping neuronal responses to a social pheromone in a *Drosophila* model' - I use a neuronal imaging technique to map some of the major neurons that flies use to perceive bee and possibly other pheromones. Based on the neural stimulus-dependent staining

that I used, I present three olfactory receptor neurons - Or-49b, Or-56a and Or-98a - as potentially active in flies to the perception of bee pheromone. If *Drosophila* as a pre-social model uses receptors similar to those of the honey bee itself then our best candidates yet from the honey bee will be the functional homologues to these three genes, which are: *Am*Or-4, -14, -51, -78, -94 and -160. Finally, in this chapter I summarise the results of my two empirical chapters to illustrate the benefits and limitations of *Drosophila* as a model in sociobiology. I also suggest possible extensions of the present work to help future research build upon these results and bridge the knowledge gap surrounding social discontinuities between pre-social and eusocial species.

4.2 *Drosophila* as a model for eusocial insects

While *Drosophila* do interact with other individuals in the population for mating (Villella and Hall 2008) as well as feeding (Wu et al. 2003) they lack the behavioural characteristics that define them as truly social in sociobiological terms. Despite not having a rich social behavioural repertoire, there is precedence in using *Drosophila* as a model for *Apis* response to queen pheromone. Sannasi (1969) first found that adult female fruit flies had reduced ovary size comparable to that of worker bees when exposed to a single component of honeybee queen pheromone (9-ODA). Since then work in the Thompson lab has demonstrated that *Drosophila* females exposed to synthetic QMP develop smaller ovaries that contain fewer mature eggs

then did untreated controls (Camiletti et al. 2013). QMP also reduces the number of adult offspring produced after mating. Additionally, fruit flies polymorphic at the *foraging* locus act differently when exposed to QMP (Camiletti et al. 2014). The 'rover' genotype was less responsive to QMP and was therefore comparable to forager bees where the 'sitter' genotype was more responsive to queen pheromone, similar to nurse bees. Last, using RNAi, olfactory receptors -49b, -56a and -98a were suggested to be responsible for pheromone perception in the fly itself (Camiletti et al. 2016).

This series of studies raised several questions that I have addressed in this thesis. First, while the above studies focused on females, I showed that male *Drosophila* are akin to drones in that they are attracted to pheromone and increase mating effort toward conspecific females (Chapter 2). This study used both a dual-choice T-maze and courtship assay to discover how QMP influenced male flies. These assays were used as they are simple to perform, informative in nature and have well developed protocols (Tully and Quinn 1985; Manning 1960). Both behavioural assays have been used to study a wide array of odours including ethanol (Lee et al. 2008; Schneider et al. 2012), DEET (Lee et al. 2010) and even *Drosophila melanogaster* specific pheromone (11-*cis*-vaccenyl acetate; Kurtovic et al. 2007; Farhan et al. 2013) but to my knowledge this is the first eusocial pheromone used in either assay. Second, my use of the fluorescent neuronal labeling technique (NFAT) indicated that *Drosophila* likely perceive QMP through stimulation of olfactory neurons located

within the basiconic sensilla on the antenna (Chapter 3). Following the Camiletti et al. (2016) study, I further confirmed that Or-49b, Or-56a and Or-98a are likely responsible for the perception of QMP in flies as they fluoresced in the presence of QMP where Or-19a and Or-85a did not. While this novel NFAT system has been used to identify DEET (Kain et al. 2013) and *Drosophila* cuticular chemical extracts sensing neurons (Masuyama et al. 2012) my study shows that it can be used with an even wider array of stimuli. Like single-sensillum recordings (de Bruyne et al. 2001), which has been the standard for olfactory odor coding in *Drosophila*, I have further demonstrated that this NFAT system may be a useful alternative in any odor based olfactory study.

Thus far, it seems that a pheromone that has evolved a diversity of functions in *Apis*, can elicit a similar range of behaviours in *Drosophila*. This certainly raises the prospect that *Drosophila* can be used in conjunction with honey bee QMP as a model to identify and reconstruct some of the molecular machinery that regulates a reproductive division of labour within social insect societies. Continuing to use these types of comparative approaches with established model organisms, like *Drosophila*, can be used to support theoretical models like the ground plan hypothesis that serve to address the evolutionary origins of social behaviour.

4.3 Future experimental strategy

While the research contained in my thesis has certainly added to comparative understanding of how flies respond to queen mandibular pheromone, more can be done to develop the fruit fly as a model for *Apis*. Building on the studies presented here could help further drive research in the honey bee itself. First, while we showed that male *Drosophila* akin to drones show significant attraction to queen pheromone (Chapter 2), it would be equally interesting to see how female flies respond to QMP in a dual choice assay. Would they predictably be repulsed by QMP as we know its limits their reproductive potential (Camiletti et al. 2013) or would they act in a homologous fashion to retinue workers who show steadfast attraction to the pheromone (Free 1987)? Further, I would build on the Camiletti et al. (2014) study showing how the two polymorphs of the foraging gene, sitters (*forS*) and rovers (*forR*), are differentially sensitive to ovary deactivation with queen pheromone much like their bee counterparts nurses and foragers. Within the T-maze it would be expected that the sitters like young workers would have a higher affinity for QMP over the rovers acting as a pseudo-replacement for older forger bees.

While my NFAT study (Chapter 3) demonstrated that flies likely use Or-49b, Or-56a and Or-98a to perceive QMP, this system can certainly be utilized further. The next step of this study should further confirm my results through dissecting the brain and analyzing the antennal lobe for fluorescence to QMP. It would also be of interest to

study higher levels of the brain like mushroom bodies as they may also become excited by QMP. If this the case, perhaps the newly emerging connectome of the fly brain could provide further insight into a fly's perception of queen pheromone (Chiang et al. 2011). Further, an open-access database, named FlyCircuit (http://www.flycircuit.tw), has been constructed for online archiving, analysis, and visualization of all the collected neurons and could be compared to for any future studies. Finally a technique developed by Gonzalez-Bellido and Wardill (2012) allows for neuronal imaging within thick invertebrate tissue samples such as a *Drosophila* thorax and abdomen. If this was done using the NFAT system in flies it could provide a full neuronal body view of QMP perception in the fly from the primary olfactory centers of the antenna, to the brain and across the body - to ovary innervation in females for example.

Queen mandibular pheromone is made up of five major components: 9-oxo-2-decenoic acid (9-ODA), two enantiomers of 9-hydroxy-2-decenoic acid (9-HDA), 4-hydroxy-3-methoxyphenylethanol (HVA) and methyl-p-hydrobenzoate (HOB). These components work synergistically causing the large-scale behaviour and physiological changes seen in *Apis mellifera* workers and drones. These components have been studied with a wide variety of bee behaviours and, therefore, are appealing to future comparative assays using the fruit fly. First, 9-ODA is an effective attractant over large distances and elicits highly predictable responses in flying drones (Gary and Marston 1971). This component could be tested alone with

male flies in the same T-maze and courtship arena and again compared to a drone response. Further, both enantiomers of 9-HDA and HOB have been shown synergize with 9-ODA to increase male attraction at close range (Brockmann et al. 2006; Loper et al. 1996) which could be further looked into with flies in both behavioural assays.

Within the honey bee the actual receptors responsible for QMP perception have yet to be functionally identified. It is widely accepted that 9-ODA is perceived through the olfactory receptor *Am*Or11 (Wanner et al. 2007) but the other components have yet to be understood this well. By utilizing the NFAT system with each of the individual QMP components I suggest specific olfactory receptors can be teased out against each component of QMP. This could add to the rough map I have suggested and provide further detail where future honey bee studies could begin.

4.4 Conclusion

The end goal of the comparative methods purposed is ultimately to test the genes I implicate in the fly on the honey bee itself. While the bee is not fully amenable to genetic manipulation as is *Drosophila*, it is hopeful that future research may allow for more direct genetic manipulation. Using the novel and dynamic CRISPR/Cas9 (Hale et al. 2012) system may soon allow for specific genomic editing on the genome of the honey bee (Ben-Shahar 2014). Further, improved transformation techniques should allow for better future integration of RNAi elements, which thus-far have had

less than perfect success (Jarosch and Moritz 2011). Using new techniques like these should allow comparative studies, like mine, to verify the function of homologous genes.

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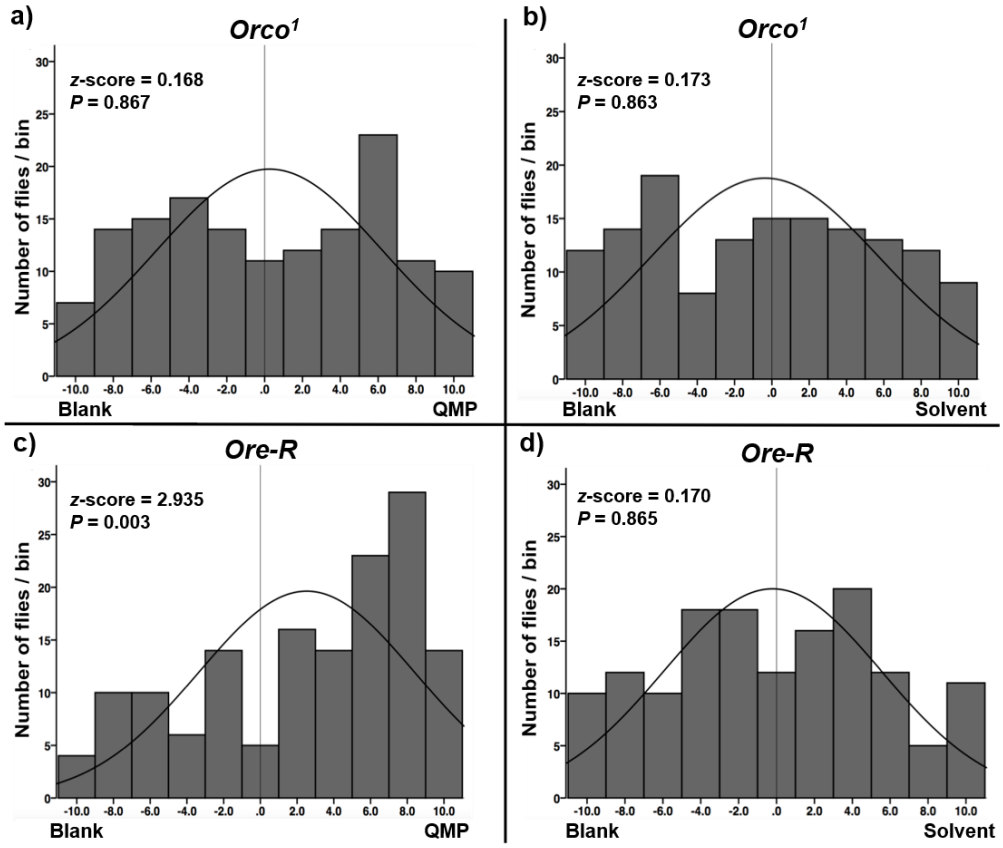

Figure B.1. Virtual frequency histograms of male flies within T-maze chambers. The four plots show the final distribution of flies among ± 1 cm bins for treated (**a**, **c**) and control (**b**, **d**) trails. *Orco¹* mutants are uniformly distributed (Kolmogorov-Smirnov test statistic for uniformity, $P > 0.05$) and thus show no bias towards either end of the T-maze, regardless of QMP. *Ore-R* flies, by contrast, deviate from a uniform distribution in the direction of QMP (K-S test for uniformity, $P < 0.001$). We superimpose a normal curve simply to help visualize departures from centrality.

Equation B.1. The formula for the area of a polygon. This equation calculates the area under the line for each individual second, subsequently allowing for the determination of the absolute sum of the entire graph.

$$\left| \frac{(x_1 y_2 - y_1 x_2) + (x_2 y_3 - y_2 x_3) \ldots\ldots + (x_n y_1 - ynx_1)}{2} \right|$$

Appendix C: Chapter 3 Supplemental Information

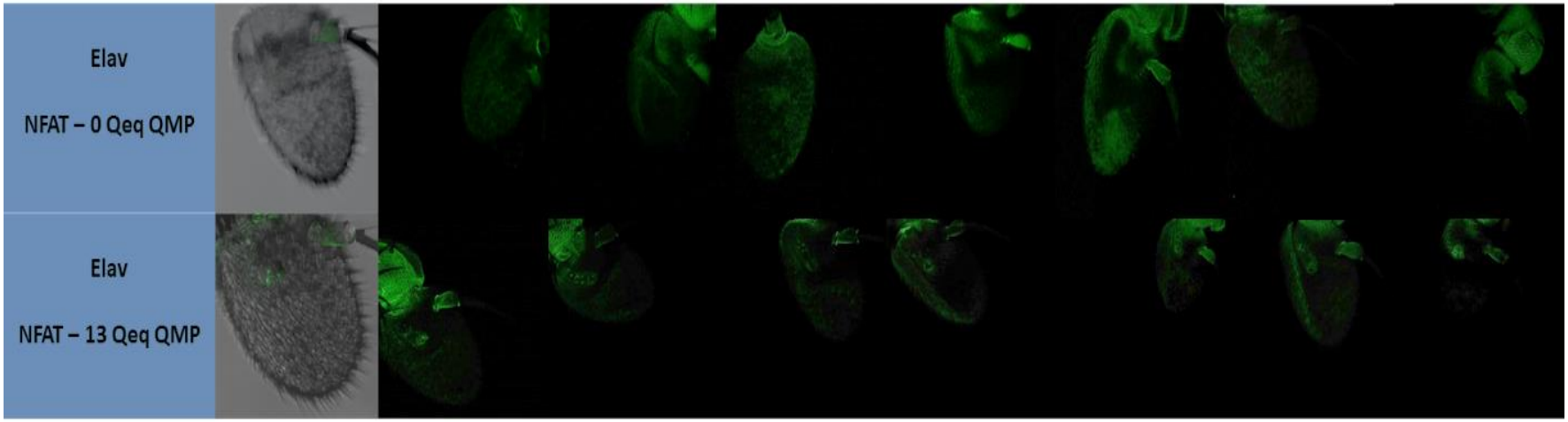

Figure C.1 All Confocal micrographs of antenna from neuronal-driven NFAT *Drosophila* stimulated with queen pheromone or solvent.

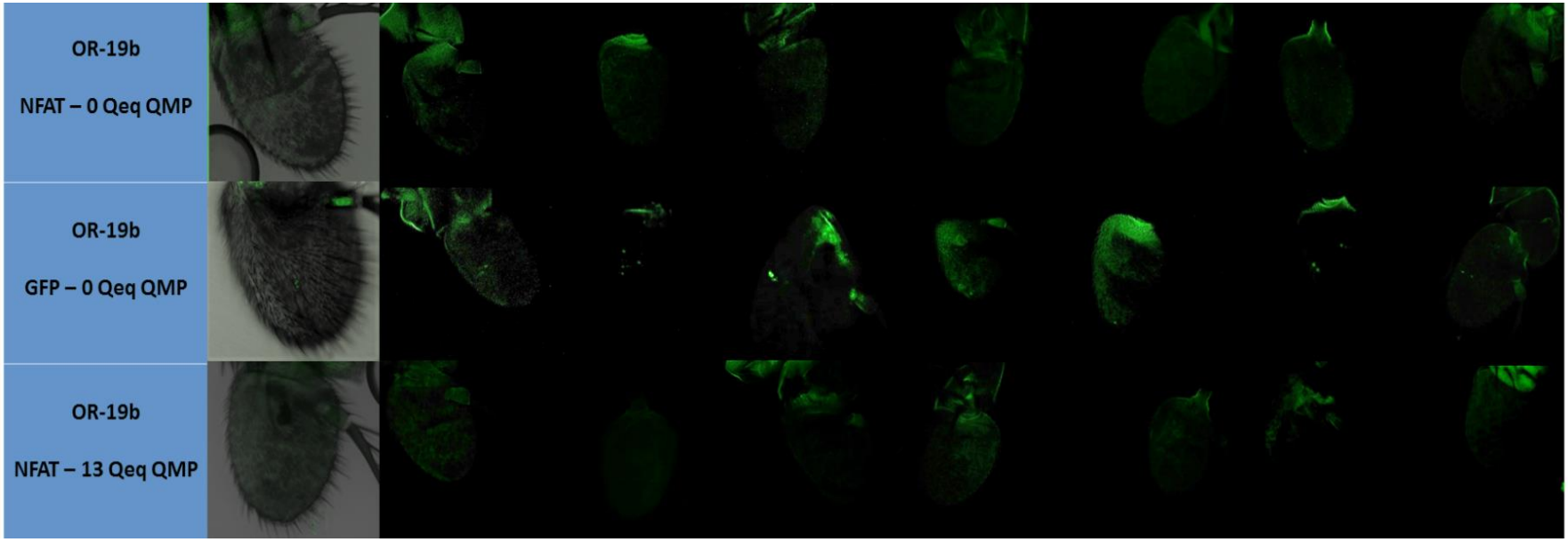

Figure C.2 Confocal micrographs of antenna of Or-19b driven NFAT *Drosophila* stimulated with queen pheromone or solvent. A neuronally driven olfactory strain is also included as a positive control.

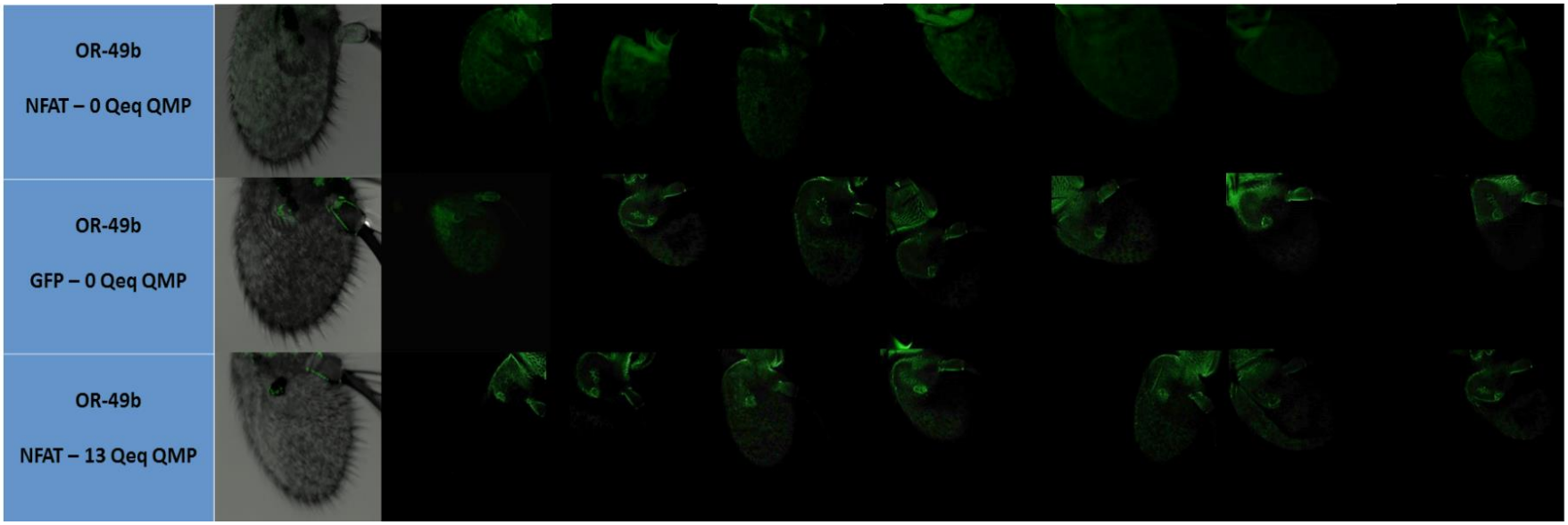

Figure C.3 Confocal micrographs of antenna of Or-49b driven NFAT *Drosophila* stimulated with queen pheromone or solvent. A neuronally driven olfactory strain is also included as a positive control.

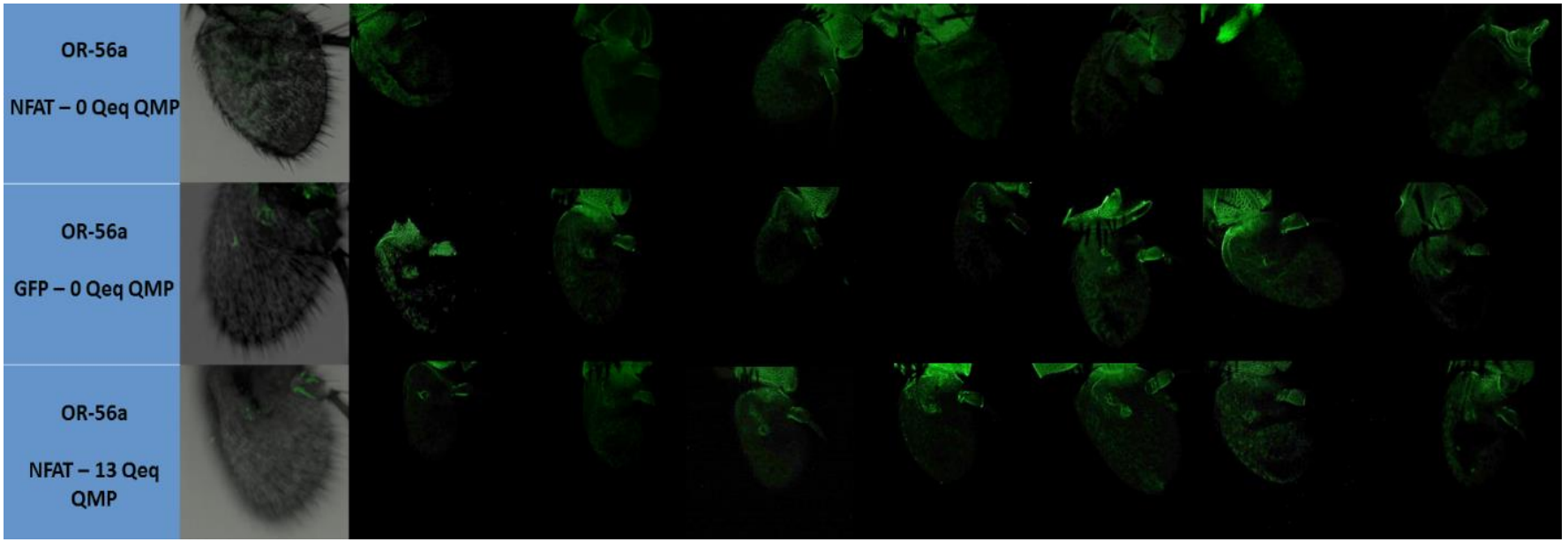

Figure C.4 Confocal micrographs of antenna of Or-56a driven NFAT *Drosophila* stimulated with queen pheromone or solvent. A neuronally driven olfactory strain is also included as a positive control.

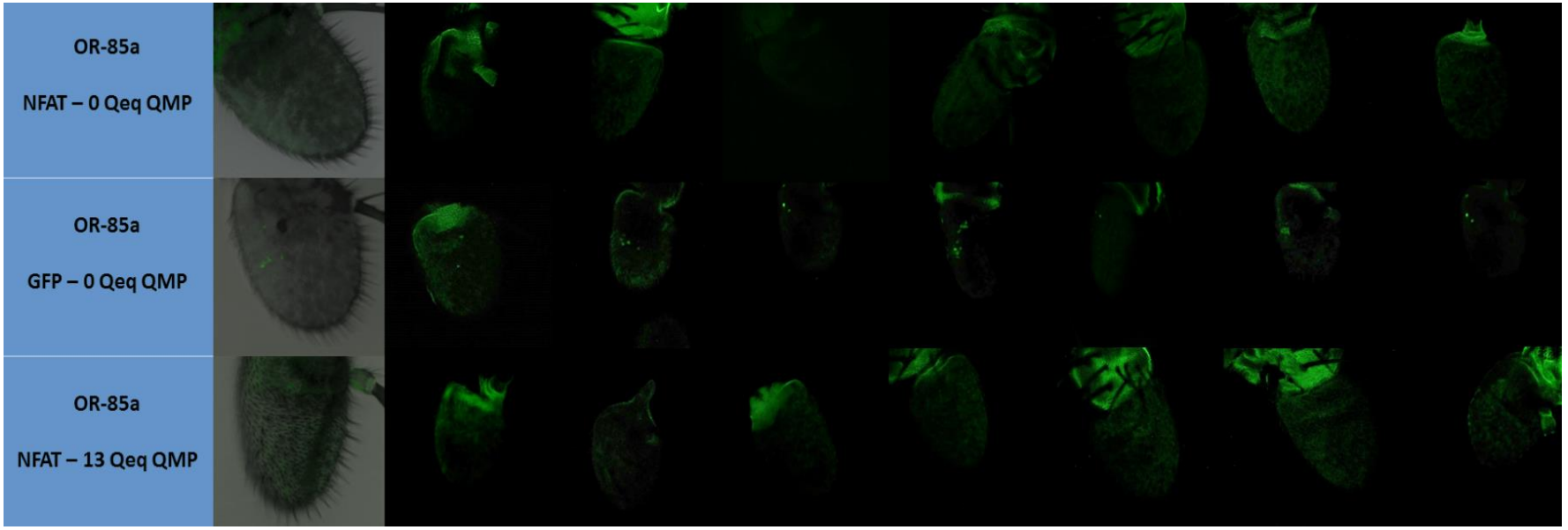

Figure C.5 Confocal micrographs of antenna of Or-85a driven NFAT *Drosophila* stimulated with queen pheromone or solvent. A neuronally driven olfactory strain is also included as a positive control.

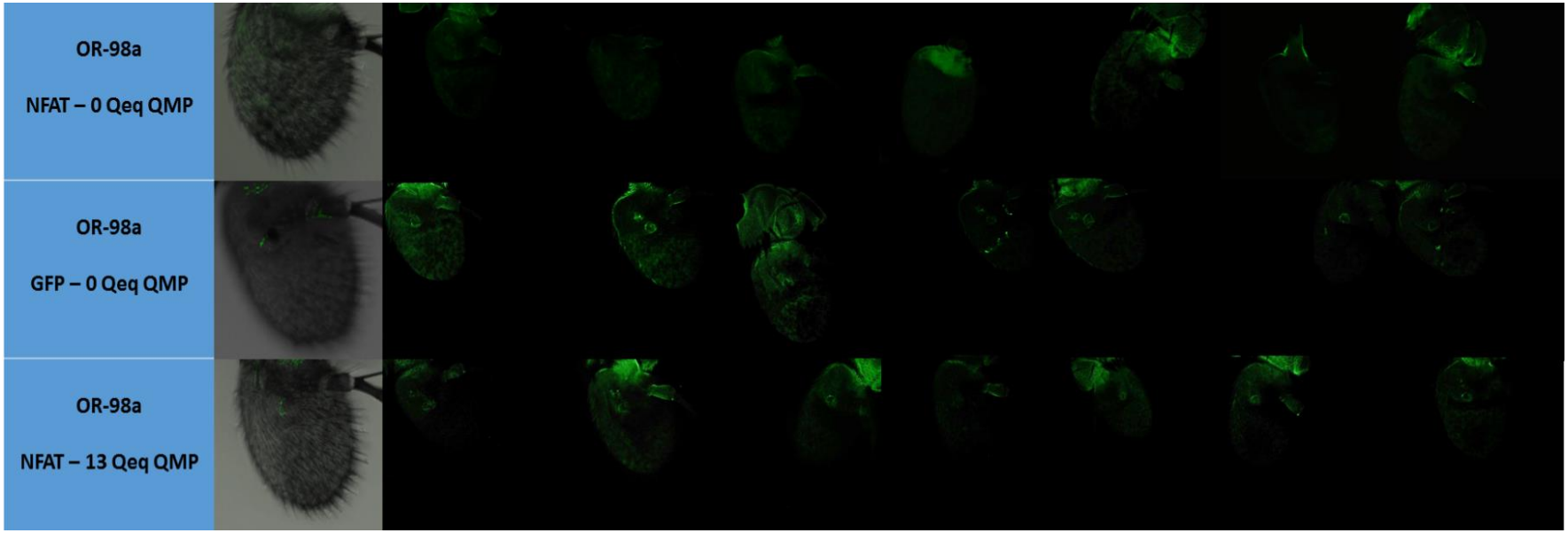

Figure C.6 Confocal micrographs of antenna of Or-98a driven NFAT *Drosophila* stimulated with queen pheromone or solvent. A neuronally driven olfactory strain is also included as a positive control.

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