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Genetic components to worker sterility in the honey bee, Apis mellifera

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Genetic components to worker sterility in the honey bee, *Apis mellifera*

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ABSTRACT

The primary characteristic that defines eusocial species is reproductive division of labour. Honey bee (*Apis mellifera*) colonies typically have a single reproductive queen and thousands of sterile workers. Here, I review the factors affecting worker reproduction and then contrast the brain gene expression of workers considered either reproductively altruistic (sterile) or selfish (fecund) over a series of time points. I confirmed that although, theoretically, the genes that allow workers to reproduce must be expressed in order for them to do so, it is the environmental cues, such as nutrition and pheromones, that ultimately control worker reproductive status. I then identify a new set of candidate 'genes for reproductive altruism' by considering the differential gene expression of reproductive vs. sterile worker brains on each day, and over multiple consecutive time-points. It was determined that a large portion of the identified genes had metabolic functions.

Keywords: Microarray; meta-analysis; *Apis mellifera*; hymenoptera; gene ontology; ovarian development; insulin-like signaling, reproductive groundplan hypothesis, metabolic genes

STATEMENT OF CO-AUTHORSHIP

This work was performed by Alanna Backx with the help of Dr. Graham Thompson.

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LIST OF ABBREVIATIONS

9-ODA: (E)-9-oxodec-2-enoic acid
AN: Anarchist strain
BP: Brood Pheromone
Cy3: Cyanine 3
Cy5: Cyanine 5
DAVID: Database for Annotation, Visualization and Integrated Discovery
E: Environmental variable
EcR: Ecdysteroid receptor
FDR: False Discovery Rate
G: Genetic variable
GnRH: Gonadotropin-releasing hormone
GO: Gene Ontology
IIS: Insulin/insulin-like signaling
JH: Juvenile Hormone
KEGG: Kyoto Encyclopedia of Genes and Genomes
leq: Larval-equivalency units
QL: Queenless
QMP: Queen Mandibular Pheromone
QMP+: Reared with queen mandibular pheromone
QMP-: Reared in absence of queen mandibular pheromone
QR: Queenright
RGPH: Reproductive Groundplan Hypothesis
RI: Ratio x Intensity
RJ: Royal Jelly
TIFF: Tagged Image File Format
USP: Ultraspiricle
Vg: Vitellogenin

1. INTRODUCTION

1.1 An introduction to reproductive altruism and kin theory

Altruism, an action that, on average decreases the lifetime fitness of an individual (the 'actor') and benefits one or more recipients [1], has long been an evolutionary paradox. Why should an individual perform a helpful action at a cost to themselves, when it is another individual who benefits from the action? This question has led to an extensive body of theoretical and experimental literature, in which several explanations have been proposed. For example, in the *reciprocal altruism* (or *reciprocity*) theory described by Trivers, altruism can occur when repeated interactions between individuals allow an altruistic act by an individual to be repaid at a later time [2]. Cheaters who fail to reciprocate help towards the altruist may no longer receive help as a consequence, and thus reciprocally cooperative systems such as warning calls in birds [3,4] may be evolutionarily unstable if there is no kin component [2].

However, examples of so-called reciprocal altruism may better be explained by *kin selection* theory, as warning calls are costly to the actor and are often received by nearby kin, who can increase the fitness of the actor if the effect is to produce more offspring than the receiver otherwise would have [5]. Multiplied across *all* related receivers, the indirect fitness benefit to the caller can potentially compensate for any loss of direct fitness. That is, by helping relatives that, by definition, carry genes identical by descent to the actor - including the gene for calling - then this type if altruism will evolve.

1.2 Extreme altruism in eusocial insects

The indirect fitness benefits provided by kin selection can lead to the evolution of behaviours that, paradoxically, are costly to direct fitness, such as young birds that forego breeding in order to help their parents raise more offspring. Sharp *et al.* [6] demonstrated that altruistic helping in long-tailed tits (*Aegithalos caudatus*) are discriminately directed towards kin, as distinguished by individual-specific contact calls. However, the best known examples of reproductive altruism occur in social insects, in which some individuals sacrifice most, if not all, of their direct reproductive opportunities in order to help rear their siblings. Indeed, *eusocial* insect societies, in which the majority of the individuals will never become reproductively active, are among the most extreme forms of altruism seen in any species [7]. Eusociality has been described as evolving 17 times, across taxa (see Table 1.1).

Honey bees (*Apis mellifera*) belong to the order Hymenoptera, where eusociality is thought to have evolved 11 separate times [7]. All males in the Hymenopteran order are haploid [8] and it is this genomic feature that may have favoured the evolution of altruistic helping between females living in family groups, as the resulting genetic relatedness among full sisters can be very high ($r = 0.75$ vs. $r = 0.5$) [9]. Indeed, the disproportionate number of eusocial taxa within the Hymenoptera [10] suggests that genetic factors were important to Hymenopteran social evolution. Moreover, there is an expectation for genetic effects to remain important in the evolutionary

maintenance of social traits, as genes strongly contribute to social behaviour [11]. Despite the important role that gene networks and genetic variation appear to play in the origin and maintenance of social life, very few studies have tried to identify the genes involved. Moreover, we do not yet know how these genes interact within molecular and neurological pathways within a social context to regulate variation in behaviour.

Honey bees are one of the best studied examples of a eusocial insect that has a strong division of labour into reproductive and non-reproductive castes (Fig. 1.1). Honey bee colonies consist of a single reproductive female, the queen, and many thousands of sterile worker females. Under normal hive conditions, workers show a distinct pattern of age-related division of labour. Young workers perform in-hive tasks such as nursing and comb-building, while relatively older workers engage in more risky behaviour such a guarding and foraging [12]. The males – drones – are found in the hundreds at certain times during the hive 'cycle.'

1.3 Reproductive control in honey bees, and other eusocial insects

Interestingly, the queen is not defined by the possession of a special set of genes, but simply by which genes are turned on during the early developmental stages; thus queens and workers do not differ in their genome, but merely in there gene expression patterns [13-15]. It is royal jelly that is the early environmental cue that triggers the different developmental trajectories that result in the two castes [16]. There is emerging evidence that the queen phenotype is driven by epigenetic mechanisms.

Specifically, Spannhoff *et al.* [17] has suggested that (*E*)-10-hydroxy-2-decanoic acid (5% of royal jelly) contains histone deacetylase activity, which mediates epigenetic regulation of the queen genome, and thus aids in queen physiological development.

Pheromones emitted from the queen [18,19] and her brood [20,21] strongly suppress worker reproduction. Within queenright colonies, worker reproduction is exceedingly rare (< 1%) [22] but in queenless colonies a proportion of workers will activate their ovaries and begin laying unfertilized eggs that develop into males [23,24]. Worker reproduction is therefore responsive to social cues, and variation in ovary activation is best modeled as a threshold response [25]. That is, workers refrain from activating their ovaries when pheromonal signals surpass a threshold typical for queenright colonies. Below this threshold, by contrast, some workers will activate their ovaries and assume a limited reproductive role. Thus, when the inhibitory signals emitted by the queen and her brood are removed, workers are able to develop their ovaries. The mandibular glands of a mated, laying queen contain an average of 200 μg (*E*)-9-oxodec-2-enoic acid, 80 μg (*E*)-9-hydroydec-2-enoic acid, 20 μg methyl *p*-hydroxybenzoate, and 2 μg 4-hydroxy-3-methoxyphenylethanol [26]. This blend is referred to as queen mandibular pheromone, or QMP.

QMP will also inhibit the rearing of new queen pupae by workers [27], and will attract workers to the reigning queen and cause the formation of a retinue (or cluster) around the around her both within colonies and in swarms when looking for a new nest [28]. QMP will also stimulate pollen foraging and brood rearing within small

colonies [29], and influence the timing of nurse-to-forager transitions [30]. Brood pheromone, on the other hand, is comprised of 10 fatty acid esters: 0.07 μg methyl oleate, 0.03 μg ethyl oleate, 0.05 μg methyl linoleate, 0.01 μg ethyl linoleate, 0.59 μg methyl linolenate, 0.18 μg ethyl linolenate, 0.26 μg methyl palmitate, 0.09 μg ethyl palmitate, 0.26 μg methyl stearate, and 0.08 μg of ethyl stearate in one larvae. The function of brood pheromone within a hive is similar to that of QMP, as it has been shown to inhibit worker ovarian development [20,31], and inhibit queen rearing [32]. Beyond pheromones, environmental factors such as nutrition [33,34], season [35,36], and the presence of other workers [37] are known to influence worker reproductive state.

Generally, worker 'policing' will deter any cheating, even within hives where there is much genetic variation due to polyandry [38]. The main mechanism by which workers police is via selective oophagy of worker-laid eggs, while leaving most of the eggs laid by the queen. This enforces the functional sterility of their sisters [39]. A second proposed method of enforcement is though aggression towards workers with activated ovaries [38]. In both queenless and queenright colonies, workers with developed ovaries are frequently targets of aggression, while workers with inactive ovaries are not [40,41].

1.4 A practical approach to finding genes for altruism in a model system

While some aspects of eusocial behaviour are expected to have a genetic basis of control [1], the genetic architecture that underpins most social traits remains unknown. At this time, it is challenging to understand the relationship between genes and social behaviour because experimental genetic methods have yet to be developed for taxa with extreme social systems (e.g., songbirds, cichlids, and social insects). The honey bee has emerged as an important model to study the genetics of social behaviour due to the high level of sociality, the ease at which colonies are reared [42,43], and the recent availability of an on-line draft genome assembly for the honey bee [44]. This genome assembly has led to the development of a series of genomic microarrays suitable for comparative studies in behavioural genomics, as it gives a glimpse of the genome-wide expression of a model social species that varies in behaviour. Using these new tools, recent studies using honey bees have begun to explore the genetic basis of eusociality. In order to understand the key characteristic that sets eusocial species apart from other social and solitary species is the evolution of a sterile caste. In order to better describe the genetic underpinnings of such a sterile helper group, studies have focused on comparative genetic studies between groups of workers that display variation in a certain behaviour of interest.

1.4.1 Progress from queen – worker contrasts

In order to describe the genetics controlling reproductive division of labour, many have attempted to identify genes that are differentially expressed in between honey bee female castes, or between queen-destined larvae and worker-destined larvae.

Evans and Wheeler [45] identified an over-expression of metabolic enzymes in queen-destined larvae, which appears to reflect the larger growth rate of queen during the last stages of larval development. It has been hypothesized that one of the first differences in the evolutionary divergence of queens and workers was probably size [46]. In social species, large size is often an important characteristic that is correlated with dominance and increases the chances of over-winter survival [10]. An extreme example of this is the sweat bee (*Lasioglossum cinctipes*), where the two castes show little or no overlap in size. This is achieved by rearing workers and queens in temporally separate brood, which corresponds with availability of food as well as the number of workers [47]. It is thought that nutritionally-based changes in gene expression that are orchestrated through juvenile hormone and the insulin/insulin-like growth factor signaling (IIS) pathways are what cause the different developmental trajectories seen between honey bee queen and worker larvae. Specifically, developing queens up-regulate several IIS genes [48], and a knockdown of the bee ortholog of the IIS gene *target of rapamycin* (*tor*) by RNAi prevents queen development [49]. Other genes and pathways suspected of paying a role in caste differentiation and reproductive division of labour include the *major royal jelly protein* genes and *yellow* genes [50], the insulin pathway [48], and *chico* [49], as well as *vitellogenin* in honey bees [51], wasps [52], and termites [53], *hexamerin* in honey bees [54], wasps [52] and termites [55], and *CYP4AB1, CYP4AB2,* and *general protein 9* in ants [56].

Given that eusociality is based on a division of labour between fecund queens and sterile workers, these early studies focusing on comparing gene expression patterns between female castes provide only a first glimpse into the gene networks that regulate the reproductive division of labour [45,57-60]. A limitation to this approach is that queens and workers are differentiated for many morphological, behavioural and physiological characteristics, so comparing gene expression profiles is unlikely to identify the initial genes that specifically turn female ovaries on or off.

1.4.2 Progress from high – low pollen-hoarder contrasts

Natural and artificially selected traits that define certain honey bee strains have recently been utilized. For example, the amount of pollen a colony collects and stores in its brood nest can be readily quantified and selected for, resulting in high and low pollen-hoarding strains [61]. In the high pollen-hoarding strain, the average worker displays a set of behavioural and physiological traits that are different from those found in the low pollen-hoarding strain. For example, workers differ in their response to sucrose concentration [62], the average size of nectar and pollen loads collected while foraging [63], the age of initial inset of foraging [64], levels of circulating juvenile hormone in young bees [65], and differing levels of neuropeptides in the brain [66]. Amdam *et al.* [67] showed that vitellogenin, an egg-yolk precursor protein linked to oogenesis, had higher titres and transcription levels in the high pollen-hoarding strain during the first 10 days post-eclosion, relative to the low pollen-hoarding strain. They suggest that this shift in expression is causally linked to the reproductive potential in honey bee workers and that pollen hoarding seems to have a

reproductive component. Further, it has been found that the high pollen-hoarding strain has more ovarioles than those of the low pollen-hoarding strain [68]. Since a larger number of ovarioles represents a greater reproductive capacity, it was determined that female reproductive morphology, physiology and behaviour is differentially tuned during development according to levels of pollen hoarding. Genes identified though pollen-hoarding contrasts include *Mid, PIP5K, PDK1,* and *AmFor* [69,70].

1.4.3 Progress from wildtype – anarchist contrasts

A clear example of cheating behaviour has been described in the 'anarchistic' strain of honey bees [71,72]. In these colonies, workers frequently lay unfertilized, male eggs even when in the presence of a healthy, laying queen. Thus, their genetically-variable cheating allows them to activate their ovaries in the presence of inhibitory pheromones, as well as evade worker policing. It is believed that anarchistic larvae produce and emit less brood pheromone, or emit a blend that is less effective [73], and that adult workers have a higher threshold for inhibitory compounds produced by the queen and her larvae [73-75]. It has also been found that anarchist workers are less discriminatory against worker-laid eggs, as compared to queen-laid eggs, as less are removed and many worker-produced drones are reared to maturity [76].

1.4.4 Progress from A. m. capensis – A. m. scutellata contrasts

Reproductive workers are particularly well-evolved in the Cape honey bee (*A. m. capensis*), as workers have twice as many ovarioles as the workers of their closest

relatives, *A. m. scutellata*, and more than all other races [77]. Laying workers have been shown to lay quickly, within 4 days of dequeening [78]. Further, it has been demonstrated that worker policing in these hives is reduced, allowing worker-laid eggs to be reared [79]. A specific feature of the Cape bee is that workers are able to produce via thelytokous parthenogenesis [80], meaning they produce female offspring, rather than drones, through the automatic fertilisation of their eggs [81]. Thus, in a queenless colony of Cape bees, worker-laid eggs are reared until the colony eventually re-queens itself. Lattorff *et al.* [82] determined that worker thelytoky appears to be controlled by a single locus (*th*). More recently, it has been suggested that this gene plays an important role in the regulation of sterility in these bees [83]. Beyond the identification of the *th* gene, the investigation of genes controlling sterility in honey bees has been focused on wildtype, anarchist and pollen-hoarding strains. A number of candidate genes for worker sterility have also been identified through the anarchist strains, including *MRJP7, MRJP2,* and *NPC2-like* [84]. However, these genes have not been verified to show a major effect on reproductive behaviour, to date.

1.4.5 Progress from worker – worker contrasts

Most studies looking at gene expression within the honey bee worker caste attempt to describe the nurse-to-forager transition and worker sterility. Many of the genes identified related with foraging are associated with locomotory behaviour. In the honey bee, *Adar* and *Innexin 2* [85], *Tctp* and *PepIII* [86], and *Inos, Cah1, Hsc70cb, Bm-40-spa, Zormin, Smd3, Orc1, Ef2b, Sh3beta, Rfabp, Fax,* and *Mmpp2* [87] are

correlated with worker nurse-to-forager transitions. However, perhaps the most well known gene associated with foraging is the *Amfor* gene, identified by Ben-Shahar *et al.* [88]. *Amfor* is the honey bee ortholog of the *foraging* (*for*) in *Drosophila*, which has two forms, which determined whether fly larvae are 'sitters' or 'rovers.' Rovers have high levels of *for* mRNA and collect food over a larger are than do sitter flies. In the honey bee, *Amfor* is associated with the transition from in-hive worker to outside forager, which is coupled with an increase in the transcript level in the brain [88].

Candidate genes for sterility in the worker caste include, *IRS, Mid, Mlc-2, Npc2-like, PDK1, PIP5K, Trf, Ubq* [69], *Vitellogenin* and *transferrin* [89], *Anarchy1* [90], *cPLA*$_2$*, Secapin,* and phospholipase [91], *MRJP1, MRJP5, PIG-W, RpL26, PI3K* [92], and *synapsin* and *myosin* [84]. One gene in particular, *krüppel homolog 1* (*Kr-h1*), appears to be promising. Whitfield *et al.* [93] used microarrays to identify 50 genes that change in their expression level as workers transition from nurse bees to foragers, regardless of age. *Kr-h1* was identified by Whitfield *et al.* and was verified by Grozinger *et al.* [94]. It was found that *Kr-h1* is responsive to QMP treatment where it causes down-regulated young workers. However, its expression is up-regulated in older bees, suggesting that foragers may be less responsive to QMP treatment, relative to young bees [94]. *Kr-h1* is a zinc finger transcription factor that plays an important role in development and cell differentiation [95,96]. As it is highly expressed in the mushroom bodies, which integrate sensory information, *Kr-h1* may be involved in responding to chemosensory QMP stimulus that result in downstream

changes in behaviour and reproductive physiology. In general, Grozinger *et al.* [60] found that workers allowed to develop their ovaries shifted their brain gene expression to become more 'queen-like,' suggesting that a core group of gene associated with reproductive physiology.

1.5 Expression of social genes in other taxa

Despite an understanding that social behaviour should have a genetic basis, little is known about how social behaviour is mediated by the genome. Some studies have begun to identify key genes regulating social behaviour and responses. Specifically, *egr1* is a transcription-factor-encoding gene that has been discovered in multiple species. Initially a specific link to social behaviour was suggested by Mello *et al.* [97]. Social interactions in songbirds are mediated by their communication through song, which are learned vocal signals. The male zebra finch (*Taeniopygia guttata*) expresses the *egr1* gene upon hearing the song of male zebra finch that they are unfamiliar with. It was determined that previously unheard songs elicit a strong response and *erg1* expression is greatly up-regulated, while familiar songs elicit little-to-no response [97]. A familiar song likely represents a familiar individual, while an unfamiliar song may represent a threatening intruder. This *erg1* response was also found to be enhanced when the male was listening to unfamiliar calls in the presence of conspecifics, compared to when he was alone [98]. A consequence of *egr1* up-regulation is that the males become acutely territorial, which is socially relevant as a function of mating opportunity [98].

egr1 has also been determined to play a role in cichlid fish (*Astatotilapia burtoni*) dominance hierarchies, which is established and maintained through aggressive fighting and dominance in males. In this species, subordinate males have reduced fertility, and when a dominant male is removed from a group subordinate males quickly begin exhibiting dominant behaviour [99]. Shortly after this behavioural change ensues *erg1* transcription is induced in the region of the brain containing gonadotropin-releasing hormone, which is critical for reproduction [100]. Because individuals who are already dominant do not elicit this response, it is a socially-responsive change that depends on the ability to recognize social opportunity. Although *erg1* is well described, it has wide-spread effects and can suppress or enhance the transcription of other genes, depending on the cell or tissue type, little is known about how its expression affects larger gene networks within the brain [101]. The use of high-throughput, genome-wide technologies for measuring the expression of many genes simultaneously will help elucidate the complex interactions between the environment and genome in a social setting.

1.6 The goals of this thesis

The goal of my thesis was to review genetic and environmental factors affecting worker reproductive altruism, and to identify genes associated with this social behaviour. In the second chapter of my thesis, I create cohorts of reproductively sterile (altruistic) and fecund (selfish) workers using two methods in order to assess levels of worker ovarian development in both 'queenright' and 'queenless' conditions. I rear bees both in cages, where I can control nutritional and pheromonal

conditions, and also in 'split-hives,' which more closely mimic natural ecological conditions. I then review the efficacy of these two methods for affecting reproductive decision making within the context of previously published studies. In the third chapter, I analyse gene expression patterns in the brains of reproductively sterile and fecund workers, in order to examine the genetic control of reproductive decision-making in honey bees. I conclude (chapter four) by discussing my findings in the context of the evolution and control of reproductive altruism in social insects.

Table 1.1. The evolutionary frequency of eusociality resulting in sterile and fertile castes in both insect and non-insect orders.

Insect Orders	Common Names	Evolutionary Frequency of Eusociality	Reference
Hymenoptera	Ants, bees, wasps, and sawflies	11	[7]
Isoptera	Termites	1	[7]
Homoptera	Gall-forming aphids	1	[102]
Coleoptera	Bark-nesting weevils	1	[103].
Thysanoptera	Gall-forming thrips	1	[104]
Non-insects	Snapping shrimp and naked mole rats	2	[105,106]
Total		**17**	

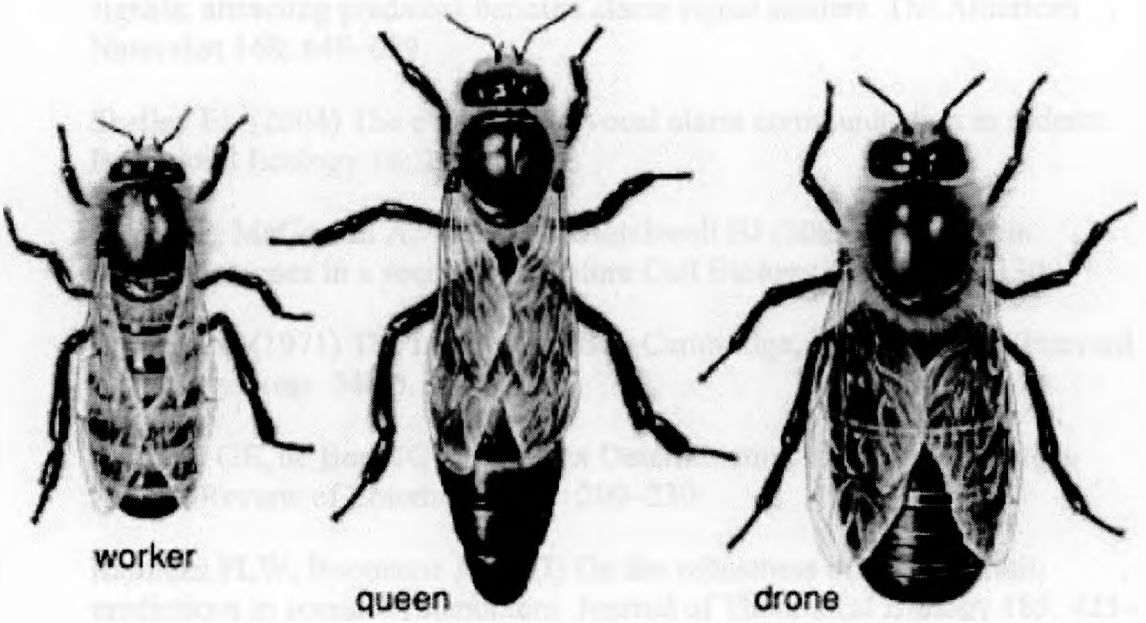


Figure 1.1 Castes of the Western honey bee, *Apis mellifera*. Adult queen, worker and drone are as labeled. The queen and drone make up the reproductive castes. The workers are the smallest in size and are generally sterile. They perform the vast majority of the tests in the hive, including foraging and caring for brood. Adapted from Encyclopædia Britannica Online (http://www.britannica.com/EBchecked/media/141787/).

References

1. Hamilton WD (1964) The genetical evolution of social behaviour. I & II. Journal of Theoretical Biology 7: 1–52.

2. Trivers RL (1971) The evolution of reciprocal altruism. The Quarterly Review of Biology 46: 35–57.

3. Platzen D, Magrath RD (2004) Parental alarm calls suppress nestling vocalization. P R Soc B 271: 1271–1276.

4. Chivers DP, Brown GE, Smith JF (1996) The evolution of chemical alarm signals: attracting predators benefits alarm signal senders. The American Naturalist 148: 649–659.

5. Shelley EL (2004) The evolution of vocal alarm communication in rodents. Behavioral Ecology 16: 169–177.

6. Sharp SP, McGowan A, Wood MJ, Hatchwell BJ (2005) Learned kin recognition cues in a social bird. Nature Cell Biology 434: 1127–1130.

7. Wilson EO (1971) The Insect Societies. Cambridge, Massachusetts: Harvard University Press. 548 p.

8. Heimpel GE, de Boer JG (2007) Sex Determination in the Hymenoptera. Annual Review of Entomology 53: 209–230.

9. Ratnieks FLW, Boomsma J (1997) On the robustness of split sex ratio predictions in social Hymenoptera. Journal of Theoretical Biology 185: 423–439.

10. Michener CD (1974) The social behavior of the bees: a comparative study. Harvard University Press. 404 p.

11. Crozier RH (2008) Advanced eusociality, kin selection and male haploidy. Australian Journal of Entomology 47: 2–8.

12. Winston ML (1991) The Biology of the Honey Bee. Harvard University Press. 281 p.

13. Foret S, Kucharski R, Pittelkow Y, Lockett GA, Maleszka R (2009) Epigenetic regulation of the honey bee transcriptome: unravelling the nature of methylated genes. BMC Genomics 10: 472.

14. Kucharski R, Maleszka R, Foret S (2008) Nutritional control of reproductive status in honeybees via DNA methylation. Science 319: 1827–1830.

15. Lyko F, Foret S, Kucharski R, Wolf S, Falckenhayn C, et al. (2010) The

honey bee epigenomes: differential methylation of brain DNA in queens and workers. Public Library of Science Biology 8: e1000506.

16. Weaver N (1966) Physiology of caste determination. Annual Review of Entomology 11: 79–102.

17. Spannhoff A, Kim YK, Raynal NJM, Gharibyan V, Su M-B, et al. (2011) Histone deacetylase inhibitor activity in royal jelly might facilitate caste switching in bees. European Molecular Biology Organisation Reports 12: 238–243.

18. Hoover SER, Keeling C, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90: 477–480.

19. Voogd S (1956) The influence of a queen on the ovary development in worker bees. Experientia 12: 199–201.

20. Jay SC (1972) Ovary development of worker honeybees when separated from worker brood by various methods. Canadian Journal of Zoology 50: 661–664.

21. Mohammedi A, Paris A, Crauser D, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L.). Naturwissenschaften 85: 455–458.

22. Ratnieks FLW (1993) Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. Behavioural Ecology and Sociobiology 32: 191–198.

23. Page RE, Robinson GE (1994) Reproductive competition in queenless honey bee colonies (*Apis mellifera* L.). Behavioural Ecology and Sociobiology 35: 99–107.

24. Velthuis HHW (1970) Ovarian development in *Apis mellifera* worker bees. Entomologia Experimentalis et Applicata 13: 377–394.

25. Barron AB, Robinson GE (2008) The utility of behavioral models and modules in molecular analyses of social behavior. Genes, Brain and Behaviour 7: 257–265.

26. Winston M, Slessor K (1992) The essence of royalty - honey-bee queen pheromone. American Scientist 80: 374–385.

27. Winston ML, Taylor OR (1980) Factors preceding queen rearing in the Africanized honeybee (*Apis mellifera*) in South America. Insectes Sociaux 27: 289–304.

28. Winston ML, Slessor KN, Smirle MJ, Kandil AA (1982) The influence of a queen-produced substance, 9HDA, on swarm clustering behavior in the honeybee, *Apis mellifera* L. Journal of Chemical Ecology 8: 1283–1288.

29. Higo H, Colley S, Winston M, Slessor K (1992) Effects of honey-bee (*Apis mellifera* L.) queen mandibular gland pheromone on foraging and brood rearing. Canadian Entomologist 124: 409–418.

30. Pankiw T, Huang Z-Y, Winston ML, Robinson GE (1998) Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. Journal of Insect Physiology 44: 685–692.

31. Jay SC (1970) The effect of various combinations of immature queen and worker bees on the ovary development of worker honey bees in colonies with and without queens. Canadian Journal of Zoology 48: 169–173.

32. Pettis JS, Higo HA, Pankiw T, Winston ML (1997) Queen rearing suppression in the honey bee – evidence for a fecundity signal. Insectes Sociaux 44: 311–322.

33. Jay SC, Jay DH (1993) The effect of kiwifruit (*Actinidia deliciosa* A Chev) and yellow flowered broom (*Cytisus scoparius* Link) pollen on the ovary development of worker honey bees (*Apis mellifera* L). Apidologie 24: 557–563.

34. Pernal S, Currie R (2000) Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L.). Apidologie 31: 387–409.

35. Lin H, Winston ML (1998) The role of nutrition and temperature in the ovarian development of the worker honey bee (*Apis mellifera*). The Canadian Entomologist 130: 883–891.

36. Hoover SER, Higo H, Winston ML (2006) Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. Journal of Comparative Physiology Biology 176: 55–63.

37. Wegener J, Bienefeld K (2009) Methods for breeding of honey bee using worker-derived drones. Zuchtungskunde 81: 265–278.

38. Ratnieks FLW (1988) Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. American Naturalist 132: 217–236.

39. Dampney J, Barron A, Oldroyd BP (2002) Policing of adult honey bees with activated ovaries is error prone. Insectes Sociaux 49: 270–274.

40. Van der Blom J (1991) Social regulation of egg-laying by queenless honeybee workers (*Apis mellifera* L.). Behavioural Ecology and

Sociobiology 29: 341–346.

41. Visscher P, Dukas R (1995) Honey bees recognize development of nestmates' ovaries. Animal Behaviour 49: 542–544.

42. Oldroyd BP, Thompson GJ (2007) Behavioural genetics of the honey bee *Apis mellifera*. Advances in Insect Physiology. 33: 1-49.

43. Robinson GE, Grozinger CM, Whitfield CW (2005) Sociogenomics: Social life in molecular terms. Nature Reviews Genetics 6: 257–270.

44. Weinstock GM, Robinson GE, Gibbs RA, Worley KC, Evans JD, et al. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931–949.

45. Evans JD, Wheeler DE (2000) Expression profiles during honeybee caste determination. Genome Biology 2: research0001.1–0001.6.

46. Wheeler DE (1986) Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. The American Naturalist 128: 13–34.

47. Knerer G, Atwood CE (1966) Polymorphism in some nearctic halictine bees. Science 152: 1262–1263.

48. Wheeler DE, Buck N, Evans JD (2006) Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera*. Insect Molecular Biology 15: 597–602.

49. Patel A, Fondrk MK, Kaftanoglu O, Emore C, Hunt G, et al. (2007) The making of a queen: TOR pathway is a key player in diphenic caste development. Public Library of Science ONE 2: e509.

50. Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R (2006) Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees. Genome Research 16: 1385–1394.

51. Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, et al. (2007) Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proceedings of the National Academy of Sciences of the United States of America 104: 7128–7133.

52. Sumner S, Pereboom JJM, Jordan WC (2006) Differential gene expression and phenotypic plasticity in behavioural castes of the primitively eusocial wasp, *Polistes canadensis*. Proceedings of the Royal Society Biology 273: 19–26.

53. Scharf ME, Wu-Scharf D, Zhou X, Pittendrigh BR, Bennett GW (2005) Gene

expression profiles among immature and adult reproductive castes of the termite *Reticulitermes flavipes*. Insect Molecular Biology 14: 31–44.

54. Evans JD, Wheeler DE (1999) Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. Proceedings of the National Academy of Science of the United States of America 96: 5575–5580.

55. Hoffman EA, Goodisman MA (2007) Gene expression and the evolution of phenotypic diversity in social wasps. BioMed Central Biology 5: 23.

56. Liu N, Zhang L (2004) *CYP4AB1, CYP4AB2,* and *Gp-9* gene overexpression associated with workers of the red imported fire ant, *Solenopsis invicta* Buren. Gene 327: 81–87.

57. Corona M, Estrada E, Zurita M (1999) Differential expression of mitochondrial genes between queens and workers during caste determination in the honeybee *Apis mellifera*. Journal of Experimental Biology 202: 929–938.

58. Barchuk AR, Cristino AS, Kucharski R, Costa LF, Simoes ZLP, et al. (2007) Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. BioMed Central Developmental Biology 7: 70.

59. de Azevedo SV, Hartfelder K (2008) The insulin signaling pathway in honey bee (*Apis mellifera*) caste development - differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. Journal of Insect Physiology 54: 1064–1071.

60. Grozinger CM, Fan Y, Hoover SER, Winston ML (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). Molecular Ecology 16: 4837–4848.

61. Page RE, Fondrk MK (1995) The effects of colony-level selection on the social organization of honey bee (*Apis mellifera* L.) colonies: colony-level components of pollen hoarding. Behavioural Ecology and Sociobiology 36: 135–144.

62. Pankiw T, Page RE (1999) The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). Journal of Comparative Physiology 185: 207–213.

63. Pankiw T, Tarpy DR, Page RE Jr. (2002) Genotype and rearing environment affect honeybee perception and foraging behaviour. Animal Behaviour 64: 663–672.

64. Calderone NW, Page RE (1996) Temporal polyethism and behavioural

canalization in the honey bee, *Apis mellifera*. Animal Behavior 51: 631–643.

65. Schulz DJ, Pankiw T, Fondrk MK, Robinson GE, Page RE Jr. (2004) Comparisons of juvenile hormone hemolymph and octopamine brain titers in honey bees (Hymenoptera: Apidae) selected for high and low pollen hoarding. Annuals of the Entomological Society of America 97: 1313–1319.

66. Humphries MA, M ller U, Fondrk MK, Page RE (2003) PKA and PKC content in the honey bee central brain differs in genotypic strains with distinct foraging behavior. Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology 189: 555–562.

67. Amdam GV, Norberg K, Fondrk MK, Page REJ (2004) Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. Proceedings of the National Academy of Sciences of the United States of America 101: 11350–11355.

68. Amdam GV, Norberg K, Page REJ, Erber J, Scheiner R (2006) Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). Behavioral Brain Research 169: 201–205.

69. Brito RM, Mchale M, Oldroyd BP (2010) Expression of genes related to reproduction and pollen foraging in honey bees (*Apis mellifera*) narcotized with carbon dioxide. Insect Molecular Biology 19: 451–461.

70. Hunt GJ, Amdam GV, Schlipalius D, Emore C, Sardesai N, et al. (2007) Behavioral genomics of honeybee foraging and nest defense. Naturwissenschaften 94: 247–267.

71. Oldroyd BP, Simolenski AJ, Cornuet JM, Crozier RH (1994) Anarchy in the beehive. Nature 371: 749.

72. Montague CE, Oldroyd BP (1998) The evolution of worker sterility in honey bees: an investigation into a behavioral mutant. Evolution 52: 1408–1415.

73. Oldroyd BP, Wossler TC, Ratneiks FLW (2001) Regulation of ovary activation in worker honey-bees (*Apis mellifera*): larval signal production and adult response threshold differ between anarchist and wild-type bees. Behavioural Ecology and Sociobiology 50: 366–370.

74. Barron AB, Oldroyd BP (2001) Social regulation of ovary activation in "anarchistic" honey-bees (*Apis mellifera*). Behavioural Ecology and Sociobiology 49: 214–219.

75. Hoover SER, Oldroyd BP, Wossler T, Winston ML (2005) Anarchistic queen honey bees have normal queen mandibular pheromones. Insectes Sociaux 52: 6–10.

76. Oldroyd BP, Ratnieks FLW (2000) Evolution of worker sterility in honey-bees (*Apis mellifera*): how anachistic workers evade policing by laying eggs that have low removal rates. Behavioural Ecology and Sociobiology 47: 268–273.

77. Jordan LA, Allsopp MH, Beekman M, Wossler TC, Oldroyd BP (2008) Inheritance of traits associated with reproductive potential in *Apis mellifera capensis* and *Apis mellifera scutellata* workers. Journal of Heredity 99: 376–381.

78. Hepburn HR, Crewe RM (1991) Portrait of the Cape honeybee, *Apis mellifera capensis*. Apidologie 22: 567–580.

79. Moritz RFA, Kryger P, Allsopp MH (1999) Lack of worker policing in the Cape honeybee (*Apis mellifera capensis*). Behaviour 136: 1079–1092.

80. Greeff JM (1996) Effects of thelytokous worker reproduction on kin-selection and conflict in the Cape honeybee, *Apis mellifera capensis*. Philosophical Transactions: Biological Sciences 351: 617–625.

81. Verma S, Ruttner F (1983) Cytological analysis of the thelytokous parthenogenesis in the Cape honeybee (*Apis mellifera capensis* escholtz). Apidologie 14: 41–57.

82. Lattorff HMG, Moritz RFA, Fuchs S (2005) A single locus determines thelytokous parthenogenesis of laying honeybee workers (*Apis mellifera capensis*). Heredity 94: 533–537.

83. Lattorff HMG, Moritz RFA, Crewe RM, Solignac M (2007) Control of reproductive dominance by the *thelytoky* gene in honeybees. Biology Letters 3: 292–295.

84. Thompson GJ, Kucharski R, Maleszka R, Oldroyd BP (2006) Towards a molecular definition of worker sterility: differential gene expression and reproductive plasticity in honey bees. Insect Molecular Biology 15: 637–644.

85. Adams HA, Southey BR, Robinson GE, Rodriguez-Zas SL (2008) Meta-analysis of genome-wide expression patterns associated with behavioral maturation in honey bees. BioMed Central Genomics 9: 503.

86. Sarma Sen M, Whitfield CW, Robinson GE (2007) Species differences in brain gene expression profiles associated with adult behavioral maturation in honey bees. BioMed Central Genomics 8: 202.

87. Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, et al. (2006) Genomic dissection of behavioral maturation in the honey bee. Proceedings of the National Academy of Sciences of the United States of America 103: 16068–16075.

88. Ben-Shahar Y, Robichon A, Sokolowski M, Robinson G (2002) Influence of gene action across different time scales on behavior. Science 296: 741–744.

89. Koywiwattrakul P, Thompson GJ, Sitthipraneed S, Oldroyd BP, Maleszka R (2005) Effects of carbon dioxide narcosis on ovary activation and gene expression in worker honeybees, *Apis mellifera*. Journal of Insect Science 5: 36.

90. Thompson GJ, Paar JR, Kucharski R, Maleszka R, Oldroyd BP (n.d.) A genetic locus for worker sterility in a social insect. Unpublished. .

91. Thompson GJ, Kucharski R, Maleszka R, Oldroyd BP (2008) Genome-wide analysis of genes related to ovary activation in worker honey bees. Insect Molecular Biology 17: 657–665.

92. Thompson GJ, Yockey H, Lim J, Oldroyd BP (2007) Experimental manipulation of ovary activation and gene expression in honey bee (*Apis mellifera*) queens and workers: Testing hypotheses of reproductive regulation. Journal of Experimental Zoology 307A: 600–610.

93. Whitfield CW, Cziko A, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302: 296–299.

94. Grozinger CM, Sharabash N, Whitfield CW, Robinson GE (2003) Pheromone-mediated gene expression in the honey bee brain. Proceedings of the National Academy of Sciences of the United States of America 100: 14519–14525.

95. Kaczynski J, Cook T, Urrutia R (2003) Sp1- and Krüppel-like transcription factors. Genome Biology 4: 206.

96. Isshiki T, Pearson B, Holbrook S, Doe CQ (2001) *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106: 511–521.

97. Mello CV, Vicario DS, Clayton DF (1992) Song presentation induces gene expression in the songbird forebrain. Proceedings of the National Academy of Science United States of America 89: 6818–6822.

98. Dong S, Clayton DF (2009) Habituation in songbirds. Neurobiology of Learning and Memory 92: 183–188.

99. Grosenick L, Clement TS, Fernald RD (2007) Fish can infer social rank by observation alone. Nature 445: 429–432.

100. Burmeister SS, Jarvis ED, Fernald RD (2005) Rapid behavioral and genomic responses to social opportunity. Public Library of Science Biology 3: e363.

101. Robinson GE, Fernald RD, Clayton DF (2008) Genes and social behavior. Science 322: 896–900.

102. Ito Y (1989) The evolutionary biology of sterile soldiers in aphids. Treand in Ecology and Evolution 4: 69–73.

103. Kent D, Simpson J (1992) Eusociality in the beetle *Austroplatypus incompterus* (Coleoptera: Curculionidae). Naturwissenschaften 79: 86–87.

104. Crespi BJ (1992) Eusociality in Australian gall thrips. Nature 359: 724–726.

105. Duffy JE (1996) Eusociality in coral-reef shrimp. Nature 381: 512–514.

106. Jarvis JU (1981) Eusociality in a mammal: cooperative breeding in naked mole-rat colonies. Science 212: 571–573.

2. DATA CHAPTER 1

Factors affecting worker sterility in honey bees: a field study and meta-analysis

2.1. INTRODUCTION

Worker sterility is a striking feature of insect sociality, and the question of how it evolved in social Hymenoptera has dominated theoretical discussions of insect sociobiology for a generation [1,2]. Though it is clear from kin selection theory that both genetic (*G*) and environmental (*E*) factors are essential for the evolution and expression of social traits [3], empirical studies are only beginning to identify specific factors on a species-by-species basis. The honey bee (*Apis mellifera*) is playing a lead role in this regard. As an emerging model in behavioural genetic research [4], we now know that environmental factors (i.e., extrinsic factors), as well as worker, queen and drone (paternal) genotype or strain (i.e., intrinsic factors), can influence the conditional expression of worker sterility via differential ovary activation [5,6]. Though worker sterility is a complex trait with behavioural, physiological and morphological components, it is most commonly quantified as a function of ovary activation, either in terms of total ovariole number or presence of developing oocytes [7,8].

Environmental effects on worker ovary activation are obvious, as pheromones emitted from the queen [9,10] and her brood [11,12] strongly suppress worker reproduction. Within queenright (QR) colonies, or those with a healthy, laying queen, worker

reproduction is exceedingly rare [13], but in queenless (QL) colonies a proportion of workers will activate their ovaries and begin laying unfertilized eggs that develop into males [14]. An exception to this is the 'anarchic' strain (see below). Likewise, brood pheromone, emitted from developing worker larvae will signal to the adult workers within the hive that a healthy, laying queen is present, and inhibit worker ovarian activation [15]. The effects of brood pheromone are, however, less important in maintaining worker sterility. Worker reproduction is therefore responsive to social cues, and variation in ovary activation is often modeled as a threshold response [16]. That is, workers refrain from activating their ovaries when pheromonal signals drop below a threshold typical for queenright colonies. Below this threshold some workers will activate their ovaries and assume a limited reproductive role. Beyond pheromones, environmental factors such as nutrition [17,18], season [19,20], and the presence of other workers [21] are known to influence worker reproductive state. QL workers reared on a high protein diet had higher levels of ovarian development than did their sisters reared on a low-protein diet [17]. Furthermore, QL workers reared in the summer months more readily activated their ovaries than did their QL sisters during the cooler seasons [22], and the presence of fecund, egg-laying workers inhibited the ovaries of younger QL workers [23].

On the genetic front, the direct effect of individual genotype on the propensity to activate ovaries is apparent from colony patrilines where the threshold response of workers sired by specific drones vary in their level of ovary activation upon dequeening [24] – or even

while she is present, a condition described as worker reproductive 'anarchy' [25]. Presumably, these patrilines segregate by alleles that respond to social circumstance and influence ovary activation. The 'anarchic' strain, characterised by workers that activate their ovaries and lay eggs despite the presence of a functional queen [26], highlights the role of genotype in the conditional expression of sterility. Not only do anarchic (egg laying) workers belong to particular patrilines within single colonies, but they also respond to artificial selection, indicating significant additive genetic variation for egg laying behaviour [27]. In effect, the anarchist lines reveal additive genetic variation for sterility, similar to other honey bee strains that have responded to selection for high and low worker reproductive rates [28].

No doubt both *G* and *E* factors are important for the regulation of worker sterility within colonies, as might interactive effects between genetic and environmental factors - *i.e.*, *G* x *E* effects. To this point, however, we do not know the relative magnitude of these effects on worker ovary activation scores. We, therefore, do not know the extent to which 'nature' versus 'nurture' explains variation in ovary activation rates in honey bee societies. One approach to generating a quantitative estimate of these effects is to systematically gather published test statistics that estimate *G* or *E* effects on ovary activation, and conduct a meta-analyses to estimate a family-wide effect size for both types of factors [29].

In this study, I use a meta-analysis to review published studies that explicitly measure worker ovary activation as a function of *G* or *E* manipulations, and I provide a first-order estimate of the environmental versus genetic effect on this important social trait. Meta-analysis is an increasingly popular method for summarizing individual studies within a standardized quantitative framework [30]. In this analysis I include my data from two novel field studies that measure ovary activation as a function of the presence or absence of either real or synthetic queen mandibular pheromone (QMP), which is the key signal, produced by the queen, that inhibits ovarian activation.

2. METHODS

2.2.1 Field studies

I conducted two independent field experiments in the spring of 2010, manipulating an environmental variable known to strongly suppress ovary activation rates in workers – namely, queen mandibular pheromone (QMP). In the *seeded-cage experiment*, I controlled the presence of so-called PseudoQueens (Contech Enterprises Inc., Victoria Canada) within bee cages. These pheromone-emitting devices are used to mimic the presence of a queen within a hive. In the *split-hive experiment*, I controlled the presence of a live pheromone-producing queen within single-brood chamber colonies via a split-hive design.

To set-up both of these experiments, I collaborated with the Honey Bee Research Centre at the University of Guelph to raise standard colonies within Langstroth hives in the spring of 2010 (Field colonies #24, #25, #37, #155 and #291). Each colony contained a healthy queen of standard commercial Buckfast stock that was allowed to mate with several closely related drones (provided by E. Guzman, University of Guelph).

To manipulate levels of ovary activation among workers I exploited the QMP signal that controls ovarian development in workers within natural colonies. Workers reared within queenright (QR) colonies almost invariably have inactive ovaries [31][23], while those reared under queenless (QL) conditions will tend to develop their ovaries and lay

unfertilised eggs. For both the split-hive and seeded-cage experiments, I therefore reared workers under effectively QR or QL conditions, as described below.

2.2.2 Seeded-cage experiment (Experiment 1)

To generate cohorts of QR and QL workers in cages, I removed large numbers (n > 2000) of late-stage pupae from 3 inbred colonies, and incubated them overnight (32°C, 70% R.H.) in an incubator (LabLine Imperial II Incubator, Labline Instruments Inc., Melrose Park, Illinois). Upon emergence the following morning, for each of the 3 colonies I collected 180 one-day-old workers and placed them into 6 cages (approx. size 20 cm x 15 cm x 15 cm) each with 30 workers. Three cages contained a dummy PseudoQueen that contained no QMP (hereafter 'QMP-'), while the other 3 contained an active PseudoQueen impregnated with QMP (hereafter 'QMP+'). These PseudoQueens release approximately one queen-equivalent (> 0.1 μg/bee) of QMP per day. For each source colony, I established three sets of cages (3 QMP+ and 3 QMP-), for a total of 18 cages seeded with a grand total of 540 workers. I provided each cage with a mix of royal jelly, honey and water (9:9:2) and replaced food and water daily. After 14 days I collected all surviving workers by flash-freezing in whole cages in liquid nitrogen.

2.2.3 Split-hive experiment (Experiment 2)

To generate cohorts of QR and QL workers in living hives, I collected emergent adult workers as described above. Immediately following collection of brood frames, which contain pupating workers, I physically split 5 source colonies into QR and QL halves, and

paint-marked 200 individual workers according to colony-half of destination (water-based paint marker, Sharpie). I re-introduced the marked same-age workers into the QR or the QL section of their natal colony. Each split section consisted of one brood box containing a series of 5-6 frames; a honey frame, two-to-three brood frames (depending on make-up of source colony), an empty frame, and a second honey frame. I included an empty frame to reduce the unused space in the hive to limit honey robbing, or the stealing of honey by another colony. In addition to physically separating the split-halves by no less than 15 m, I minimized cross-contamination between split halves by reducing the entrances, fixing queen excluders below the brood box on all QL hives (to prevent entry of a new queen), and introducing roughly equal amounts of nurse bees for all hives. The splits were created in the early morning before older workers began their daily forage. During this process, all hives were transported to a new bee-yard to reduce forager 'confusion,' caused by assimilation to an area. After 13 days, I collected all paint-marked workers by flash-freezing them in liquid nitrogen. Any 'drifted' individuals (of which only a couple were found), representing cross-contamination, were ignored.

2.2.4 Ovary dissections and statistical analysis

To score the level of ovary activation I dissected individual bees to examine left and right ovaries under a stereoscope (Nikon SMZ 15002). I assigned an ovary activation score based on the single most developed ovary (or ovariole), using a 5-point scale. A score of '0' represented completely inactive, vestigial ovaries with undefined ovarioles, while a

score of '4' indicated highly developed ovaries with mature eggs (Table 2.2.1). To avoid scoring bias, I remained blind to the experimental treatment of each bee.

To measure the effect of pheromone treatment on the level of worker ovary activation, I ran a generalized linear model regression in which I specified ovary Score as the response variable. The predictor variables were Treatment, Colony, and Replicate (Cage) for Experiment 1, and Treatment and Colony for Experiment 2. Because the data was not normally distributed (Shapiro-Wilk W Test, W = 0.817, $P < 0.0001$, performed in JMP v7.0) the model was run with a specified Poisson distribution using the Design (v2.3) and Car (v2.0.1) packages in **R** statistical software (*http://cran.r-project.org/*; v 2.12.2). I calculated *F*-statistics for all Treatments, and all possible interactions.

Table 2.2.1. Scheme for scoring the level of ovary activation in worker honey bees. These criteria were developed by Hoover *et al.* [20] and consider three characters: i) the mean size of ovarioles, ii) the total number of ovarioles, and iii) the presence of developed eggs within ovarioles.

Ovary Score	Ovary Description
0	Undeveloped: completely resting and thread-like, small ovarioles not easily separated
1	Oogenesis Starting: ovaries slightly swollen, but egg cells cannot be distinguished from nutritive cells
2	Slight Development: ovarioles slightly 'bumpy,' egg and nutritive cells can be distinguished, nutritive cells larger than egg cells
3	Moderate Development: ovarioles 'bumpy,' egg cells larger than nutritive cells
4	Highly Developed: at least one ovarioles contains a fully mature ovum

2.2.5 Meta-data set collection

My meta-data set is based on a directed search of the literature until December 2010, to identify as many of the published empirical studies as possible (ideally, all) that measured conditional effects on ovary activation in workers. I used a keyword search in Web of Knowledge (http://apps.isiknowledge.com) using a combination of the following key words: honey bee, honeybees, *Apis*, *mellifera*, *mellifica*, ovarioles, ovary, ovarian, development, activation, egg-laying, queen substance, reproduction, pheromone. In addition, I conducted secondary searches using forward and reverse citation links for each paper, as well as author searches. This secondary effort uncovered additional studies not identified from keywords alone. Because of the low success for solicitation of unpublished results, only published studies were included in the analysis [29]. The results of this literature survey are shown in Table 2.2.2. It should be noted that not all studies listed were included in the meta-analysis.

To be included in the meta-analysis, studies had to satisfy the following criteria: (i) the test result had to be quantified using a statistic (e.g., *r, t, F,* χ^2, *P*) that could be converted to effect size; (ii) a control group had to be clearly identified; (iii) the study had to report on European strains of honey bee (*Apis mellifera mellifera*) and not other subspecies; (iv) the sample size of each group had to be provided; and (v) ovarian development must have been scored or quantified explicitly, whether it be through ordinal scores or a binary *on/off* categorization.

From each published study, I identified the experimental factors tested against ovary activation scores, and broadly categorised these factors into 'environmental' or 'genetic' categories. Environmental factors included pheromonal manipulations [9-11,20,23,32-35], nutrition manipulation [17,19], seasonal observations [20], and miscellaneous factors such as carbon dioxide narcosis [5,36]. Likewise, genetic factors included natural mutants or strains [6,37], response to selection experiments [38,39], and pedigree analysis [40]. Some studies report the effects of multiple *G* or *E* test variables within a single publication, in which case the appropriate test statistics were extracted and listed independently in Table 2.2.2.

2.2.6 Meta-analysis model

Because the studies identified from my bibliometric search are compiled from different sources, they are not *a priori* standardized in their response scale. For example, some studies report ovary activation as a function of oocyte development, either in binary [41-43] or multi-state scales [9,19,20,32,33]. Other studies record the degree of ovary activation, and thus potential to egg-lay, as a function of ovariole number but without reference to oocytes [37,39,44]. Because of this heterogeneity in response data, typical for meta-analyses, I calculated the corresponding effect size (a measure of the strength of the relationship between two variables) of each study's statistic using MetaEasy software (http://www.jstatsoft.org/v30/i07/) [45]. To estimate effect size using this program, I first extracted summary statistics from each publication according to the

guidelines described by Field *et al.* [29]. Specifically, I noted sample size, and where provided, the mean and standard deviation. I also extracted any and all test statistics.

From sample size information, and different combinations of other summary statistics, I calculated a standardised effect size as estimated by Hedge's *g* [46] for each test. For the vast majority of studies, calculation of *g* was straightforward; it is readily estimated from any conventional test statistic [47]. In other cases, for example, when only exact *P*-values were provided (without accompanying test statistics), they were used to obtain an approximate *t*-value using the appropriate degrees of freedom. Exact or approximate *t*-values can then be used to calculate the mean difference (or difference in means between two groups). Even if exact *P*-values were not provided, an approximate *P*-value can be estimated from the significance (alpha) level reported by the study. For example, if "$P < 0.01$" alone was reported, the *P*-value was considered to be exactly 0.01. However, using this estimation method, comparisons deemed non-significant ($P > 0.05$) that did not provide any other descriptive statistic or mean values (nor standard deviation) were excluded. This exclusion of study data potentially introduces bias into the meta-analysis, but is unavoidable. Finally, if no descriptive statistics were available, the study was also excluded. All of these procedures are explained in detail in the Cochrane Handbook for Systematic Reviews of Interventions (v4.2.6) [47]. The majority of the studies tested for more than one factor (i.e., equivalent to many small studies within one publication), and thus the total number of effect size calculations used in the meta-analysis exceeds the number of published studies.

For estimating *g* I used the DerSimonian and Laird (DL) procedure, which is arguably the simplest and most commonly used method for fitting a random effects model to meta-analysis data [48]. Specifically, it assumes there is a distribution of treatment effects and utilizes the observed effects from individual studies to estimate this distribution [49]. Cochrane's *Q* and I^2 were also calculated from summary statistics. *Q* is analogous to the goodness-of-fit test in logistic models, and can be used to test for heteogeneity between studies. In general, if the 95 % CI for the effect sizes of individual studies do not overlap, then significant inter-study heterogeneity is inferred. Similarly, I^2 describes the proportion of total variance that is due to between-study heterogeneity, as opposed to sampling error. In comparison to *Q*, an I^2 value greater than 50% indicates substantial between-study heterogeneity [47].

Table 2.2.2 Summary of published studies considering ovarian development in European honey bee workers. Both "Environmental" and "Genetic" factors are considered, and are broken down into descriptive subheadings. Statistics are summarized from each noted study where available, along with a brief description of the main finding. Unless otherwise stated, all studies are in relation to a non-treatment control group within the European honey bee subspecies. Bolded statistics were included in model.

Factor	Results of Investigation	Statistic	Ref
1.0 Environmental			
1.1 Pheromonal			
1.1.1 QMP	Queen and synthetic QMP inhibit queen cell building in hives	-	[50]
1.1.1.1 Real QMP	Queen extract in food inhibits ovaries	**$P < 0.04$**	[51]
	Queen extract inhibits ovaries	**$P < 0.001$**	[51]
	Presence of queen inhibits ovaries in cages	**$P < 0.001$**	[52]
	9-ODA inhibits caged worker ovaries	**$P < 0.001$**	[52]
	Presence of queen inhibits ovaries in cages	-	[8]
	Queen extract inhibits ovaries	-	[35]
	Queen effect continues to inhibit ovaries two weeks after dequeening	-	[11]
	Presence of queen inhibits ovaries in hives	-	[53-55]
	Presence of queen larvae, pupae had no effect on ovary inhibition relative to mated-QR hive	-	[53]
	Mated queen in cages inhibits ovarian development of CO_2-treated and control workers	**$F = 75.00$, $P < 0.001$**	[56]
	QL workers had greater ovarian development and a greater terminal oocyte size than the QR workers	**$P < 0.001$**	[1]
	QL workers reintroduced into QR hives had significant ovarian regression relative to workers kept in QL conditions	**$P < 0.001$**	[1]

Factor	Results of Investigation	Statistic	Ref
	Workers reared with queens had lower ovary activation than bees in QL cages	**$Z = -9.04$, $P < 0.0001$**	[57]
	Workers reared with queens with removed mandibular glands had lower ovary activation than bees in QL cages	**$Z = -9.34$, $P < 0.0001$**	[57]
	Presence of queens with mandibular glands caused inhibition of ovaries	**$Z = -0.737$, $P = 0.5$**	[57]
	Queen extract inhibits ovaries, relative to worker extract	-	[10]
1.1.1.2 Synthetic QMP	Synthetic QMP inhibits ovaries	**$P < 0.01$, χ^2 test**	[42]
	Synthetic QMP inhibits ovaries	-	[50]
	Synthetic QMP inhibits AN and WT ovaries	**$F_{3,28} = 13.70$, $P < 0.0001$**	[33]
	Synthetic QMP inhibits ovaries	**$F_{3,69} = 48.24$, $P < 0.0001$**	[9]
	Synthetic QMP inhibits ovaries	**$F_{4,25} = 27.67$, $P < 0.0001$**	[9]
1.1.2 Brood Pheromone	Synthetic BP inhibits ovaries by contact	**$\chi^2 = 24.9$, $P < 0.001$**	[41]
	Synthetic BP inhibits ovaries by diffusion	**$\chi^2 = 10.4$, $P < 0.01$**	[41]
	Synthetic BP inhibits ovaries by ingestion	**$\chi^2 = 16.8$, $P < 0.001$**	[41]
	Synthetic BP inhibits AN and WT ovaries	**$F_{3,24} = 7.58$, $P = 0.001$**	[32]
	QR hives with brood inhibited ovaries relative to QR and QL hives without brood	-	[11]
	Presence of brood in QR hive inhibited ovaries	-	[34]
	Presence of worker brood inhibits ovaries in QL hives	-	[58]
	Presence of unsealed brood negatively correlated with ovary development in swarmed colonies	$r^2 = 0.477$, $P < 0.01$	[59]
	Unsealed brood and eggs inhibit ovaries in QR hives	**$P < 0.05$**	[60]
	BP extract inhibits ovary development	**$P = 0.01$**	[61]

Factor	Results of Investigation	Statistic	Ref
	Colonies without larvae showed ovarian development and signs of swarming (queen cells)	-	[7]
	Extracts from brood inhibited ovaries	-	[62]
	E-β-ocimene extract from larvae (instars 2-3) inhibit ovaries of caged workers	**$Z = -2.168$, $P = 0.0301$**	[63]
	Experimental conditional threshold for preventing ovarian development corresponded to 600 leq of ethyl palmitate and 47 leq of methyl linolenate (components of BP)	**$P < 0.01$, χ^2 test**	[12]
1.1.3 Other Workers	Laying workers inhibit ovaries of other workers	-	[34]
	Laying workers inhibit ovaries of other workers	-	[23]
	Presence of narcotised workers in QL cages inhibited ovarian development of untreated workers	**$U = 19.5$, $P < 0.001$**	[64]
1.2 Seasonal	Seasonal effect on ovary scores, more development during summer vs. spring and fall	**$F_{6,178} = 45.99$, $P < 0.001$**	[20]
	More ovary development in spring vs. summer and fall in QR hive	$P < 0.01$	[65]
	Ovaries more developed in early fall than in late summer	$P < 0.01$	[65]
	No difference between average ovariole number between "strong" and "weak" colonies	-	[22]
1.2.1 Temperature	Low temperature inhibits ovary development in cages	**$P < 0.01$**	[19]
1.2.2 Swarming	Greater ovarian development during swarming than prior to swarming	-	[59]
	Swarming colonies had greater ovary development than non-swarming colonies	-	[66]
1.3 Per Annum	Higher ovary scores in 2002 than 2003	**$F_{1,178} = 4.15$, $P = 0.04$**	[20]

Factor	Results of Investigation	Statistic	Ref
	Effect of year on ovarian development	Wald stat = 26.9, $P < 0.001$	[6]
1.4 Nutritional			
1.4.1 Protein	Effect of adult and larval diet on ovary scores	**$F_{3,44} = 25.11$, $P = 0.001$**	[20]
	Greater ovary development with pollen from high vs. low protein kiwifruit flowers	**$P < 0.01$**	[17]
	Honey enriched in RJ increased ovary activation	**$P < 0.01$**	[19]
	Ovarian development effected by type of pollen fed to workers	**$F = 10.39$, $P < 0.0001$**	[18]
	Protein correlated with ovary development	**$r^2 = 0.601$, $P < 0.001$**	[18]
	Protein consumption correlated with ovary development	**$r^2 = 0.905$, $P < 0.0001$**	[18]
	Honey with protein supplement resulted in greater ovary development	-	[66]
	Significant difference between ovarian development of three treatments: royal jelly and honey, pollen and honey, and pollen and sucrose.	**$\chi^2 = 35.47$, $P < 0.001$**	[21]
1.5 Other			
1.5.1 CO_2 Narcosis	Treatment inhibited ovaries in caged workers, 8 days old	**$G = 17.3$, $P < 0.001$**	[5]
	Treatment inhibited ovaries in caged workers, 10 days old	**$G = 20.8$, $P < 0.001$**	[5]
	Treatment inhibited ovaries in caged workers, 12 days old	**$G = 7.6$, $P < 0.02$**	[5]
	Treatment inhibited ovaries in caged workers	**$G = 35.6$, $P < 0.001$**	[36]
	Treatment had no effect on ovaries after 4 hours	**$\chi^2 = 0.069$, $P = 0.8$** **$\chi^2 = 1.077$, $P = 0.6$**	[36]
	Treatment had no effect on ovaries after 24 hours	**$\chi^2 = 0.271$, $P = 0.6$**	[36]

Factor	Results of Investigation	Statistic	Ref
	Treatment had effect on ovaries after 48 hours	**$\chi^2 = 16.831$, $P = 0.001$**	[36]
		$\chi^2 = 7.735$, $P = 0.021$	
	Treatment had effect on ovaries after 96 hours	**$\chi^2 = 10.764$, $P = 0.013$**	[36]
	Treatment affected ovaries and egg development	**$P < 0.05$**	[64]
	Narcosis inhibited ovarian development of caged workers (QR and QL)	**$F = 75.00$, $P < 0.001$**	[56]
	Narcosis reduced the proportion of workers with eggs in their ovaries	**$\chi^2 = 27.7$, $P < 0.001$**	[67]
1.5.2 Presence of Comb	Worker comb in cages increased ovary development	-	[68]
1.5.3 X-ray Irradiation	Radiation delayed ovary activation	-	[69]
1.5.4 Quercetin	Workers treated with quercetin (0.01%) had greater ovarian development than controls	**$P < 0.001$**	[70]
1.5.5 Age of Worker	Young workers showed greater ovarian development compared to older bees	**$\chi^2 = 78.2$, $P < 0.001$**	[21]
		$\chi^2 = 368.7$, $P < 0.001$	
2.0 Genetic			
2.1 Response to Selection			
2.1.1 Pollen Hoarding	Ovarian development affected by strain	$\chi^2 = 18.38$, $P < 0.0001$	[38]
	Greater number of ovarioles in high vs. low pollen hoarding strains	-	[39]
	Pollen-hoarding strain had more ovarioles	**$P < 0.001$**	[44]
2.1.2 Anarchists	AN ovaries active in QR WT hive	**$\chi^2 = 5.13$, $P = 0.023$**	[26]
	AN ovaries more active than WT, in QL hive	**$\chi^2 = 21.21$, $P < 0.001$**	[26]
		$\chi^2 = 6.34$, $P = 0.011$	
		$\chi^2 = 14.32$, $P < 0.001$	
		$\chi^2 = 14.09$, $P < 0.001$	

Factor	Results of Investigation	Statistic	Ref
	AN ovaries not inhibited in QR AN hive	**$\chi^2 = 13.26$, $P < 0.001$**	[26]
		$\chi^2 = 11.77$, $P < 0.001$	
	AN ovaries more active than WT in QR hive	**$P_{AN\text{-}Q} < 0.01$**	[32]
		$P_{WT\text{-}Q} < 0.012$	
	No effect of queen type (AN or WT) on ovaries of AN or WT workers	**$P_{AN} = 0.133$**	[32]
		$P_{WT} = 0.685$	
	WT foragers have more ovarioles than AN foragers in Summer	**U = 1,949.5, $P < 0.001$**	[37]
	WT foragers have more ovarioles than AN foragers in Spring	**U = 2,442, $P = 0.009$**	[37]
	WT non-foragers have more ovarioles than AN non-foragers in Summer	**U = 2533.5, $P = 0.02$**	[37]
	AN-backcross workers developed ovaries 20% of time in QR hive	-	[27]
	Greater ovary development in AN bees in WT QR hive	**$\chi^2 = 24.8$, $P < 0.001$**	[43]
	Greater ovary development in AN bees in WT QR hive	**$\chi^2 = 17.7$, $P < 0.001$**	[43]
	Greater ovary development in AN bees in WT QR hive	Wald stat = 82.0, $P < 0.001$	[6]
	AN workers more likely to develop ovaries in AN host, relative to WT host	**$P < 0.001$**	[71]
		$P = 0.009$	
2.2 Genotype	Effect of subspecies on worker ovaries between *A. m. ligustica* and *A. m. adansonii*	$F = 14.416$, $P < 0.05$	[72]
	No subspecies effect on ovarian development in QL cage	-	[68]
	Total ovariole number higher in *A. m. adansonii* then in *A. m. ligustica*	$F_{1,22.1} = 8.89$, $P = 0.0069$	[73]

Factor	Results of Investigation	Statistic	Ref
	Worker-destined individuals not affected by rapamycin/FK506 pharmacology, either in developmental time or ovariole number	$F_{2,117} = 0.51$, $P = 0.51$ $F_{2,41} = 1.68$, $P = 0.20$	[74]
2.3 Patriline	Worker patriline affects ovary activation	-	[75,76]
	One and two weeks after de-queening some subfamilies had higher frequency of development	$P_{wk\text{-}1} = 0.003$, $P_{wk\text{-}2} = 0.031$	[75]
	In microhives containing three different patrilines: unequal sharing of reproduction seen	$F_{1,2} = 5.72$, $P = 0.02$	[24]
	Worker patriline affects laying rates	-	[13]
2.4 Age-Related	Higher ovary scores in AN non-foragers in Summer	**U = 2,209, $P < 0.001$**	[37]
	Higher ovary scores in AN non-foragers in Spring	**U = 2,769, $P < 0.001$**	[37]
	Higher ovary scores in WT non-foragers in Summer	**U = 2,602, $P < 0.001$**	[37]
	Positive correlation between ovariole number and ovary development in non-foragers	**r = 0.135, $P = 0.01$**	[37]

NB: QMP, queen mandibular pheromone; 9-ODA, (E)-9-oxodec-2-enoic acid; BP, brood pheromone; QR, queen right; QL, queenless; RJ, royal jelly; AN, anarchist strain; WT, wild type; JH, juvenile hormone; leq, larval-equivalency units (calculated per bee, per day).

2.3. Results

2.3.1 Field study

In both field experiments the workers held in presence of queen pheromone had a significantly lower mean ovarian score than those held without pheromone (Fig. 2.3.1-2.3.2). In the seeded-cage experiment the differences were very pronounced for all three colonies, with no significant differences between source colonies ($F_{3,211}$ = 0.697, P = 0.405, Treatment × Colony) or between cage replicates ($F_{5,210}$ = 0.173, P = 0.678, Treatment × Colony × Cage). Therefore, I combined all data, and the presence of QMP resulted in workers with significantly less developed ovaries than those held without QMP ($F_{1,212}$ = 5.008, P = 0.026). It should be noted that there was considerable worker mortality in the cage experiment, as compared to the split-hive experiment, in general, with the highest mortality seen in QMP+ group from colony #155. The higher mortality seen in one experiment as compared to the other is likely due to the stresses related to being caged.

In the split-hive experiment those workers held with the queen had significantly less ovarian development than those from the same hive but separated from the queen in all 5 hives (Fig. 2.3.2). Furthermore, the overall scores were lower than those of workers kept in cages. However, there was a much greater between-hive variability observed than in the seeded-cage protocol ($F_{5,1281}$ = 23.126, P = 1.7e-06, Treatment × Colony). In addition, mortality of marked workers was somewhat lower.

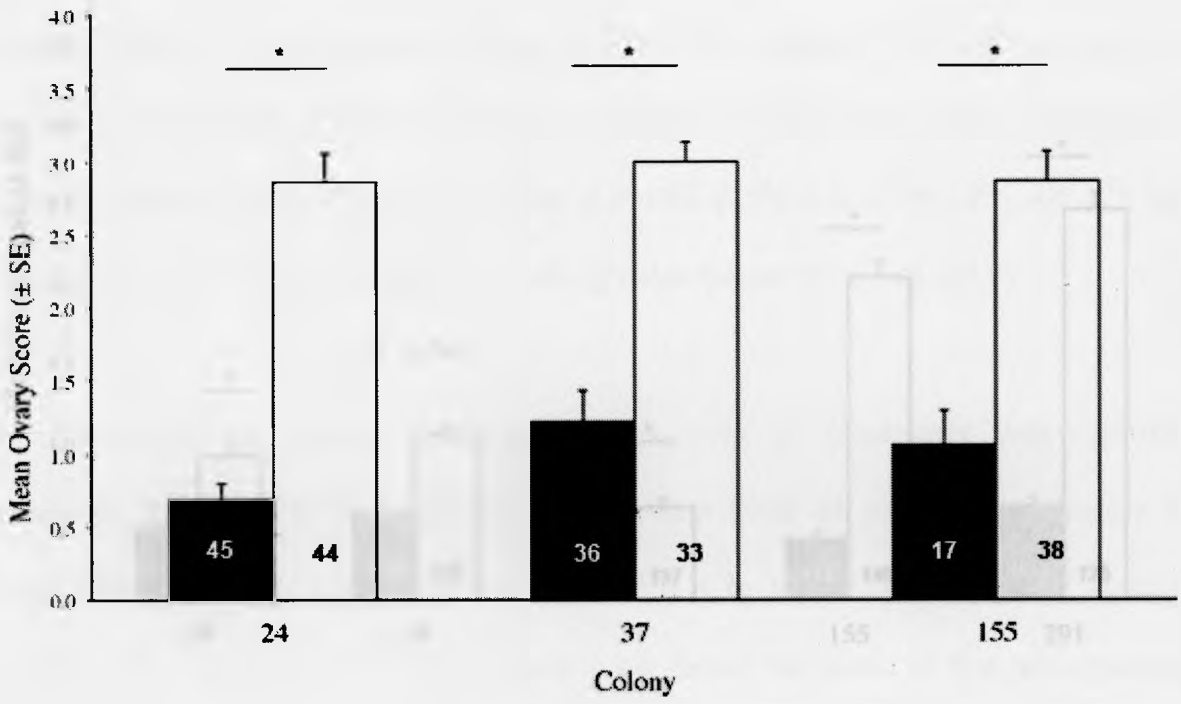


Figure 2.3.1 Results of 'seeded cage' experiment (Experiment 1) ovary microdissection assay. Workers were reared with (QMP+), or without (QMP-), QMP. Black bars represent the mean ovary-score of QMP+ workers, and the white bars represent the mean ovary score of the QMP- workers. The numbers indicate corresponding sample size for each group.
NB: * indicates $P < 0.001$

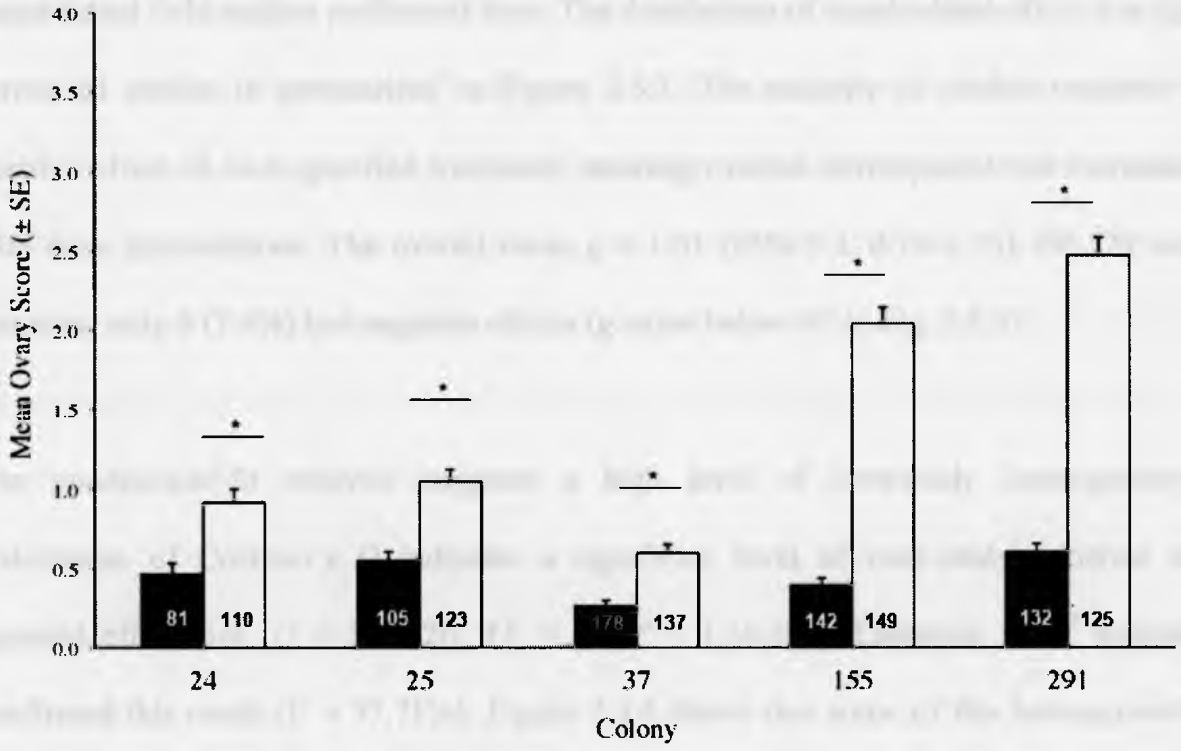


Figure 2.3.2 Results of 'split-hive' experiment (Experiment 2) ovary microdissection assay. Workers were reared in hives with (QR) or without (QL) their natal queen. Black bars represent the mean ovary-score of QR workers, and the white bars represent the mean ovary score of the QL workers. The numbers indicate corresponding sample size for each group.
NB: * indicates $P < 0.001$

2.3.2 Meta-analysis

I identified 36 studies that met the criteria for inclusion. An additional 23 studies were identified and on-topic, but were excluded from the analysis due to reasons outlined in section 2.2.5. This meta-dataset represents a disparate set of studies published between the calendar years of 1926 and 2010. I also include in the meta-analysis the two unpublished field studies performed here. The distribution of standardised effect size (g) across all studies is summarized in Figure 2.3.3. The majority of studies reported a positive effect of each specified treatment; meaning ovarian development was increased with most interventions. The overall mean g = 1.01 (95% C.I. 0.76–1.25). Of 129 test statistics, only 8 (7.4%) had negative effects (g value below '0' in Fig. 2.3.3).

The goodness-of-fit analysis suggests a high level of inter-study heterogeneity. Calculation of Cochran's Q indicates a significant level of inter-study variation in reported effect size (Q = 1573.20, d.f. = 36, P = 1.2e-307). Likewise, the I^2 statistic confirmed this result (I^2 = 97.71%). Figure 2.3.4 shows that some of this heterogeneity may come from publication bias, whereby the largest effect sizes tend to be associated with the largest standard errors (i.e., associated with the smallest sample sizes). The single largest effect (g = 7.84) also has the largest standard error (S.E. = 0.62) and appears to represent an outlier (n = 20 in each of two groups tested), as it is well outside the 95% CI and is nearly double the next-largest effect. Excluding this outlier, the mean effect size is g = 0.83 (95% C.I. 0.62–1.03, n = 128).

If individual *g*-scores are linked to their original study, and thus classified as genetic or environmental (Table 2.2.2), then we can estimate the mean effect size for *G* and *E* factors separately (Fig. 2.3.5). From this analysis, I estimate the mean *E*-effect to be strong (g = 0.97, 95% C.I. 0.72–1.22, n = 103 scores) and significantly larger than the mean *G*-effect (g = 0.36, 95% C.I. 0.09–0.63, n = 25 scores), as evidenced by the non-overlap in their respective 95% C.I. (Fig. 2.3.6). It should be noted that the excluded study was categorized as "genetic," and upon its exclusion the *G*-effect was lowered from g = 1.32, which is higher than the *E*-effect, to g = 0.36. From the published data available, the *E*-effect is therefore more than twice that of the *G*-effect. This quantitative result highlights the importance of environmental context and is consistent with our understanding that functional sterility in honey bee workers is an environmentally – mostly, socially – responsive trait.

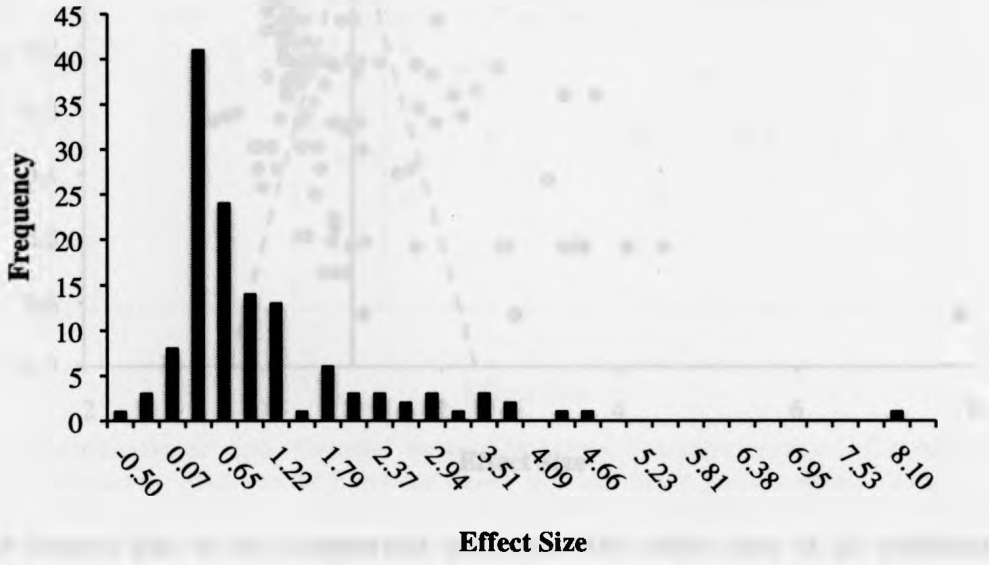


Figure 2.3.3 Frequency distribution of effect sizes. Effect sizes of all published studies considering European honey bee worker ovarian development were calculated from given statistics.Very few studies with negative effects were published between 1929 and 2010. This has created a skew in the data, which appears to have a Poisson distribution.

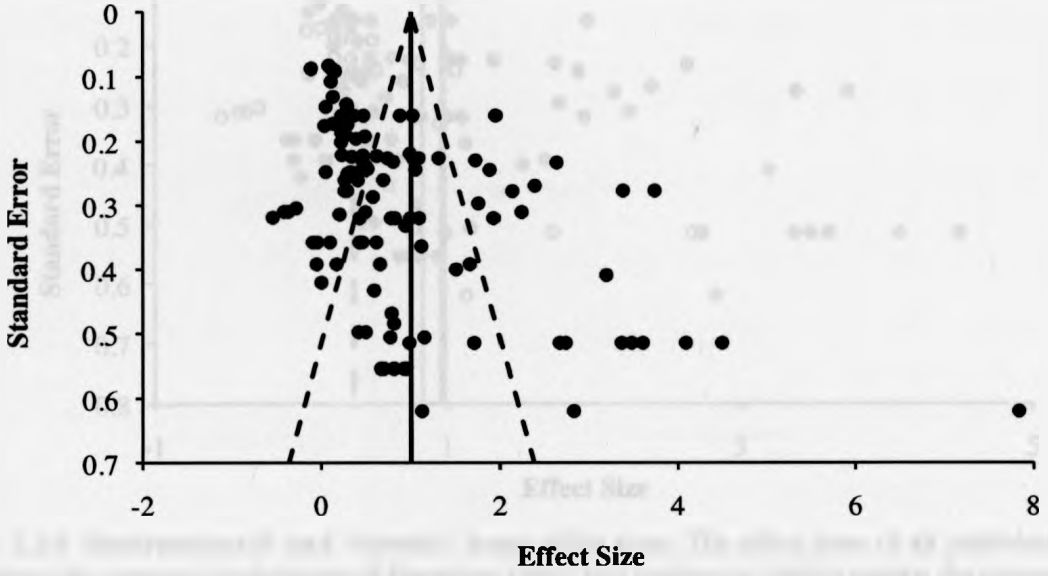


Figure 2.3.4 Funnel plot of all summarised outcomes. The effect sizes of all published studies considering the ovarian development of European honey bee workers is plotted against the corresponding standard error. The vertical solid line represents the mean effect size of the studies. The broken lines represent the 95% C.I. for the mean effect estimate. A single published test statistic represents an obvious outlier.

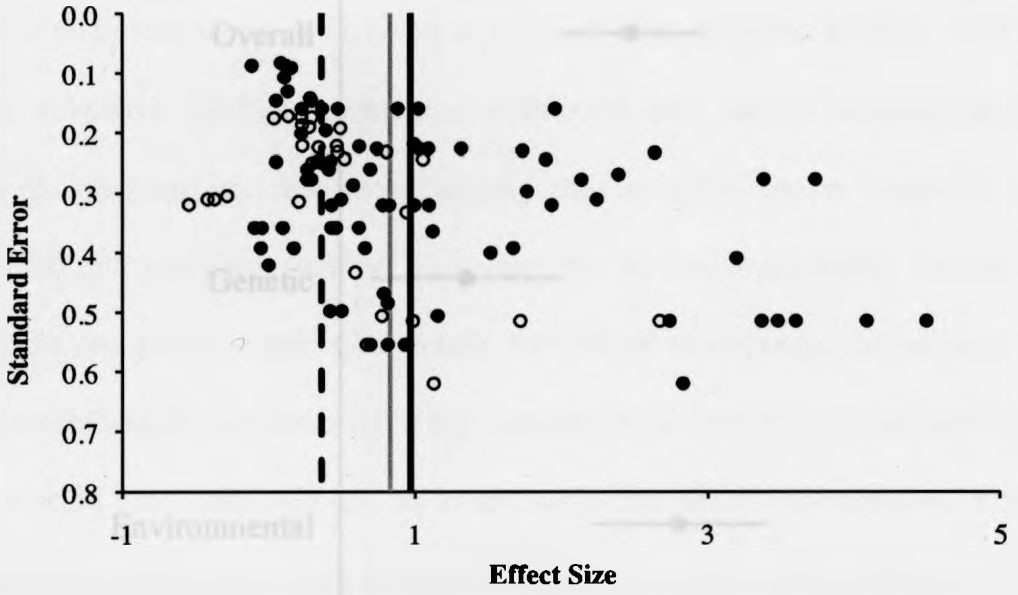


Figure 2.3.5 'Environmental' and 'Genetic' mean effect sizes. The effect sizes of all published studies considering the ovarian development of European honey bee workers is plotted against the corresponding standard error. The studies considering the effects of environmental variables on ovarian development are displayed as closed circles, with their mean effect size displayed as a solid line. The studies considering genetic factors are displayed as open circles with their mean effect size displayed as a broken line. The grey vertical line represents the mean effect size considering all studies inclusively (with the exception of the single outlier, not shown).

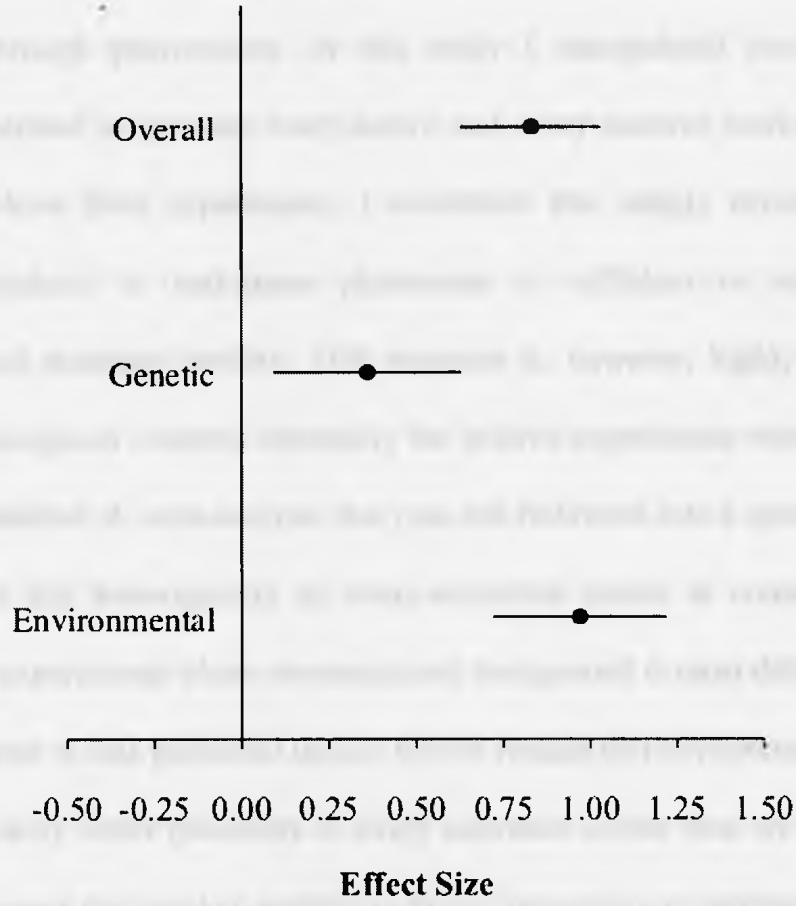


Figure 2.3.6 Forrest plot of *Genetic* and *Environmental* effect sizes and 95% C.I. in comparison to combined effect size ("Overall"). Effect sizes were calculated using all published studies (excluding the outlier) manipulating ovarian development in European honey bee workers.

2.4. Discussion

A conspicuous feature of honey bee social biology is the division of labour between reproductive queens and functionally sterile workers. The sterility of workers is conditional, however, and is highly sensitive to social context, especially to queen signal as mediated through pheromones. In this study I manipulated social context via pheromone treatment to generate ovary-active and ovary-inactive workers. Using both cage and split-hive field experiments, I confirmed that simply exposing groups of workers to synthetic or real-queen pheromone is sufficient to suppress ovarian development and maintain sterility. This response is, however, highly variable when compared to unexposed controls, especially for in-hive experiments where colony-level effects are substantial. A meta-analysis that puts this fieldwork into a quantitative genetic context reveals that heterogeneity in ovary-activation scores is common, especially among in-hive experiments where environmental background is most difficult to control. The meta-analysis of data published to-date further reveals that environmental effects are, on the whole, likely better predictors of ovary activation scores than are genetic factors. This result suggests that worker sterility is highly responsive to environmental context and genetic variation on its own contributes less than does the genetic response to environmental stimuli. This responsiveness in reproductive readiness for honey bee workers underscores the conditional nature of their sterility, and showcases worker ability to shift reproductive effort into parental ('selfish' egg-layer) versus alloparental ('altruistic' helper) roles.

2.4.1 Field study

In normal queenright colonies, the environmental cue that maintains inactive ovaries in worker bees is the presence of a healthy, egg-laying queen and her brood, as signaled by queen and brood pheromone [9]. Consequently, the presence of QMP within a cage can mimic the presence of a live queen, and has been shown to suppress ovary activation in groups of workers [77]. Similarly, the data collected here showed ovaries inhibited by queen pheromone. Workers reared in cages with synthetic QMP had significantly reduced potential to lay eggs, relative to their sisters reared in control cages (Fig 2.3.1). Likewise, workers reared in the QL half of their natal colony showed a significantly higher potential to lay eggs, as evidenced by higher ovary scores, relative to their age-matched QR sisters. In this latter experiment, however, I observed a large amount of variation in ovary scores between the host colonies that was not present in cages. This result suggests that natural variation in ovary scores is subject to $G \times E$ interaction effects, whereby the genetic effect on ovary activation depends on the differences in between-hive environments. This is likely due to the presence of brood pheromone, which was not controlled for within the split-hive experiment. It is also likely that brood pheromone accounts for the lower ovarian scores seen in the workers from the split-hive experiment, relative to the workers from the cage experiment.

2.4.2 Meta-analysis

One can test for publication bias through analysis of a funnel plot of g against SE. A sample of studies with publication bias will lack symmetry within the plot because

studies with small sample sizes and small effects are less likely to be published than are studies based on the same sample size that show a large effect [29]. There is some evidence of publication bias on worker ovarian activation. As Fig. 2.3.4 shows, a disproportionate number of effect sizes are scattered to the lower right of the plot, corresponding to a large, positive effect (and a large S.E.). Conversely, the plot shows that a study with a smaller S.E. will tend to report a smaller effect. Finally, the lower left portion of the plot appears to be missing. These patterns suggest some publication bias. It is possible that genetic studies are more easily published with small effect sizes, or in the case of the outlier, with a large effect size and extremely small sample size. However, this may also be interpreted as actual variation within the population. In this case, since most of the studies reporting large effect sizes manipulated environmental factors, it is possible that response to environmental variables is extremely heterogeneous in this species.

Both Cochrane's Q and the I^2 tests for heterogeneity indicated that inter-study variation on ovary scores was significant. Because studies manipulating both *G* and *E* factors were considered for this review, it is likely their combination into a single test for heterogeneity contributed to the observed high levels of heterogeneity. However, if the goodness-of-fit analysis is re-performed on each set of studies separately, the heterogeneity remains significant in both *E*- ($I^2 = 97.37\%$) and *G*-studies ($I^2 = 83.92\%$).

Analysing *G* and *E* studies separately also permits an estimate of the *G*- vs *E*-specific effects. As Figure 2.3.6 reveals, there is no overlap in the 95% C.I.s of the two effect size

estimates. This result indicates that environmental mainpulations of ovary activation are more effective than are genetic manipulations, and that the majority of the variation reported in the literature can be explained by environmental (extrinsic), and not genetic (intrinsic) factors. The propensity for workers to activate their ovaries is therefore first and foremost an environmentally responsive trait, despite its well-known genetic underpinnings.

Further investigation into specific *E*-factors manipulated does not reveal any obvious patterns. For example, studies using synthetic QMP did not, in general, have greater or smaller effects than did manipulations using real queens. This was not the case for the *G* components, however, as the pollen hoarding strains, which have been artificially selected to prefer foraging for either nectar or pollen, rather than both [44], appeared to show a greater effect on ovary activation, than did the anarchist mutants [26,32,33] relative to their study-specific wild type controls. I also note that there are so far relatively few studies that provide valuable data on genetic effects.

It is known that an environmental trigger, or cue, depends on an organism's genotype. This knowledge offers clues in regards to the root beginnings of a causal pathway. It is thought that variation in an organism's DNA sequence antedates all other variables, including environmental and social [78]. In the case of honey bee worker reproduction, we know there is a genotypic effect [3,6,25,39,40,44], but we do not yet know the specific genes involved. By contrast, environmental cues are better studied [9,11,15,32-34,42,50,57,60,79-81], and it has been demonstrated in this study that regardless of the

underlying genes involved, genomic expression related to reproduction is moderated by the workers' environment. Recent studies have focused on the variation allowing certain workers to more readily activate their ovaries, which is likely due to variation in the pheromone threshold [25,43,71]. Understanding the brain and its genetic expression can help identify the proximal role of nervous system reactivity within the *G* x *E* interaction.

References

1. Malka O, Shnieor S, Hefetz A, Katzav-Gozansky T (2006) Reversible royalty in worker honeybees (*Apis mellifera*) under the queen influence. Behavioural Ecology and Sociobiology 61: 465–473.

2. Crozier RH (2008) Advanced eusociality, kin selection and male haploidy. Australian Journal of Entomology 47: 2–8.

3. Hamilton WD (1964) The genetical evolution of social behaviour. I & II. Journal of Theoretical Biology 7: 1–52.

4. Robinson GE, Fernald RD, Clayton DF (2008) Genes and Social Behavior. Science 322: 896–900.

5. Thompson GJ, Yockey H, Lim J, Oldroyd BP (2007) Experimental manipulation of ovary activation and gene expression in honey bee (*Apis mellifera*) queens and workers: Testing hypotheses of reproductive regulation. Journal of Experimental Zoology 307A: 600–610.

6. Thompson GJ, Kucharski R, Maleszka R, Oldroyd BP (2008) Genome-wide analysis of genes related to ovary activation in worker honey bees. Insect Molecular Biology 17: 657–665.

7. Perepelova L (1929) Laying workers, the ovipositioning of the queen, and swarming. Bee World 10: 69–71.

8. DeGroot AP, Voogd S (1954) On the ovary development in queenless worker bees (*Apis mellifica* L.). Experientia 10: 384–385.

9. Hoover SER, Keeling C, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90: 477–480.

10. Voogd S (1956) The influence of a queen on the ovary development in worker bees. Experientia 12: 199–201.

11. Jay SC (1972) Ovary development of worker honeybees when separated from worker brood by various methods. Canadian Journal of Zoology 50: 661–664.

12. Mohammedi A, Paris A, Crauser D, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L.). Naturwissenschaften 85: 455–458.

13. Visscher P (1989) A quantitative study of worker production in honey bee colonies. Behavioural Ecology and Sociobiology 25: 247–254.

14. Winston ML (1991) The Biology of the Honey Bee. Harvard University Press. 281 p.

15. Smedal B, Brynem M, Kreibich CD, Amdam GV (2009) Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*). Journal of Experimental Biology 212: 3795–3801.

16. Barron AB, Robinson GE (2008) The utility of behavioral models and modules in molecular analyses of social behavior. Genes, Brain and Behaviour 7: 257–265.

17. Jay SC, Jay DH (1993) The effect of kiwifruit (*Actinidia deliciosa* A Chev) and yellow flowered broom (*Cytisus scoparius* Link) pollen on the ovary development of worker honey bees (*Apis mellifera* L). Apidologie 24: 557–563.

18. Pernal S, Currie R (2000) Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L.). Apidologie 31: 387–409.

19. Lin H, Winston ML (1998) The role of nutrition and temperature in the ovarian development of the worker honey bee (*Apis mellifera*). The Canadian Entomologist 130: 883–891.

20. Hoover SER, Higo H, Winston ML (2006) Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. Journal of Comparative Physiology Biology 176: 55–63.

21. Wegener J, Bienefeld K (2009) Methods for breeding of honey bee using worker-derived drones. Zuchtungskunde 81: 265–278.

22. Levin MD, Haydak MH (1951) Seasonal variation in weight and ovarian development in the worker. Journal of Economic Entomology 44: 54–57.

23. Velthuis HHW, Verheijen FJ, Gottenbos AJ (1965) Laying worker honey bees: similarities to the queen. Nature 207: 1314.

24. Inbar S, Katzav-Gozansky T, Hefetz A (2008) Kin composition effects on reproductive competition among queenless honeybee workers. Naturwissenschaften 95: 427–432.

25. Oldroyd BP, Simolenski AJ, Cornuet JM, Crozier RH (1994) Anarchy in the beehive. Nature 371: 749.

26. Barron AB, Oldroyd BP (2001) Social regulation of ovary activation in "anarchistic" honey-bees (*Apis mellifera*). Behavioural Ecology and Sociobiology 49: 214–219.

27. Oxley PR, Thompson GJ, Oldroyd BP (2008) Four quantitative trait loci that influence worker sterility in the honeybee (*Apis mellifera*). Genetics 179: 1337–

1343.

28. Hunt GJ, Amdam GV, Schlipalius D, Emore C, Sardesai N, et al. (2007) Behavioral genomics of honeybee foraging and nest defense. Naturwissenschaften 94: 247–267.

29. Field AP, Gillett R (2010) How to do a meta-analysis. British Journal of Mathematical and Statistical Psychology 63: 665–694.

30. Harrison F (2011) Getting started with meta-analysis. Methods in Ecology and Evolution 2: 1–10.

31. Velthuis HHW (1970) Ovarian development in *Apis mellifera* worker bees. Entomologia Experimentalis et Applicata 13: 377–394.

32. Hoover SER, Oldroyd BP, Wossler T, Winston ML (2005) Anarchistic queen honey bees have normal queen mandibular pheromones. Insectes Sociaux 52: 6–10.

33. Hoover SER, Winston ML, Oldroyd BP (2005) Retinue attraction and ovary activation: responses of wild type and anarchistic honey bees (*Apis mellifera*) to queen and brood pheromones. Behavioural Ecology and Sociobiology 59: 278–284.

34. Jay SC, Nelson EV (1973) The effects of laying worker honeybees (*Apis mellifera* L.) and their brood on the ovary development of other worker honeybees. Canadian Journal of Zoology 51: 629–632.

35. Voogd S (1955) Inhibition of ovary development in worker bees by extraction fluid of the queen. Experientia 11: 181–182.

36. Koywiwattrakul P, Thompson GJ, Sitthipraneed S, Oldroyd BP, Maleszka R (2005) Effects of carbon dioxide narcosis on ovary activation and gene expression in worker honeybees, *Apis mellifera*. Journal of Insect Science 5: 36.

37. Oldroyd BP, Beekman M (2008) Effects of selection for honey bee worker reproduction on foraging traits. Public Library of Science Biology 6: 463–470.

38. Amdam GV, Nilsen K-A, Norberg K, Fondrk MK, Hartfelder K (2007) Variation in endocrine signaling underlies variation in social life history. American Naturalist 170: 37–46.

39. Wang Y, Amdam GV, Rueppell O, Wallrichs MA, Fondrk MK, et al. (2009) PDK1 and HR46 gene homologs tie social behavior to ovary signals. Public Library of Science ONE 4: e4899.

40. Oldroyd BP, Osborne KE (1999) The evolution of worker sterility in honeybees:

the genetic basis of failure of worker policing. Proceedings of the Royal Society Biology 266: 1335–1339.

41. Arnold G, Leconte Y, Troullier J, Hervet H, Chappe B, et al. (1994) Inhibition of worker honeybee ovaries development by a mixture of fatty-acid esters from larvae. Comptes rendus de l'academie des sciences serie III 317: 511–515.

42. Grozinger CM, Sharabash N, Whitfield CW, Robinson GE (2003) Pheromone-mediated gene expression in the honey bee brain. Proceedings of the National Academy of Sciences of the United States of America 100: 14519–14525.

43. Thompson GJ, Kucharski R, Maleszka R, Oldroyd BP (2006) Towards a molecular definition of worker sterility: differential gene expression and reproductive plasticity in honey bees. Insect Molecular Biology 15: 637–644.

44. Amdam GV, Page RE, Fondrk MK, Brent CS (2010) Hormone response to bidirectional selection on social behavior. Evolution and Development 12: 428–436.

45. Kontopantelis E, Reeves D (2009) MetaEasy: A meta-analysis add-in for Microsoft Excel. Journal of Statistical Software 30: 1–25.

46. Hedges LV, Gurevitch J, Curtis PS (1999) The meta-analysis of response ratios in experimental ecology. Ecology 80: 1150–1156.

47. Riis J (2006) Cochrane Handbook for Systematic Reviews of Interventions 4.2.6. The Cochrane Collaboration.

48. Jackson D, Bowden J, Baker R (2010) How does the DerSimonian and Laird procedure for random effects meta-analysis compare with its more efficient but harder to compute counterparts? Journal of Statistical Planning and Inference 140: 961–970.

49. DerSimonian R, Laird N (1986) Meta-analysis in clinical trials. Controlled Clinical Trials 7: 177–188.

50. Grozinger CM, Fan Y, Hoover SER, Winston ML (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). Molecular Ecology 16: 4837–4848.

51. Butler CG (1957) The control of ovary development in worker honey bees (*Apis mellifera*). Experimentia 13: 256–257.

52. Butler CG, Fairey EM (1963) The role of the queen in preventing oogenesis in worker honeybees. Journal of Apicultural Research 2: 14–18.

53. Jay S (1968) Factors influencing ovary development of worker honeybees under natural conditions. Canadian Journal of Zoology 46: 345–&.

54. Miller DG, Ratnieks FLW (2001) The timing of worker reproduction and breakdown of policing behaviour in queenless honey bees (*Apis mellifera* L.) societies. Insectes Sociaux 48: 178–184.

55. Koywiwattrakul P, Sittipraneed S (2009) Expression of Vitellogenin and Transferrin in Activated Ovaries of Worker Honey Bees, *Apis mellifera*. Biochemical Genetics 47: 19–26.

56. Harris J, Woodring J, Harbo J (1996) Effects of carbon dioxide on levels of biogenic amines in the brains of queenless worker and virgin queen honey bees (*Apis mellifera*). Journal of Apicultural Research 35: 69–78.

57. Maisonnasse A, Lenoir J-C, Beslay D, Crauser D, Le Conte Y (2010) E-beta-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*). Public Library of Science ONE 5: e13531.

58. Jay SC (1970) The effect of various combinations of immature queen and worker bees on the ovary development of worker honey bees in colonies with and without queens. Canadian Journal of Zoology 48: 169–173.

59. Kropacova S, Haslbachova H (1970) The development of ovaries in worker honeybees in queenright colonies examined before and after swarming. Journal of Apicultural Research 9: 65–70.

60. Kropacova S, Haslbachova H (1971) The influence of queenlessness and of unsealed brood on the development of ovaries in worker honeybees. Journal of Apicultural Research 10: 57–61.

61. Kubisova S, Haslbachova H, Vrkoc J (1982) Effects of fractions of larval extracts on the development of ovaries in caged worker honey bees. Acta Entomologica Bohemoslovaca 79: 334–340.

62. Kubisova S, Haslbachova H (1978) Effects of larval extracts on the development of ovaries in caged worker honey bees. Acta Entomologica Bohemoslovaca 75: 9–14.

63. Maisonnasse A, Lenoir J-C, Costagliola G, Beslay D, Choteau F, et al. (2009) A scientific note on E-beta-ocimene, a new volatile primer pheromone that inhibits worker ovary development in honey bees. Apidologie 40: 562–564.

64. Harris J, Harbo J (1990) Suppression of ovary development of worker honeybees by association with workers treated with carbon-dioxide. Journal of Apicultural Research 29: 187–193.

65. Kropacova S, Haslbachova H (1969) The development of ovaries in worker honeybees in a queenright colony. Journal of Apicultural Research 8: 57–64.

66. Tuenin TA (1926) Concerning laying workers. Bee World 8: 90–91.

67. Brito RM, Mchale M, Oldroyd BP (2010) Expression of genes related to reproduction and pollen foraging in honey bees (*Apis mellifera*) narcotized with carbon dioxide. Insect Molecular Biology 19: 451–461.

68. Jay SC (1975) Factors influencing ovary development of worker honeybees of European and African origin. Canadian Journal of Zoology 53: 1387–1390.

69. Wegener J, Zautke F, Hoecht S, Koehler B, Bienefeld K (2006) Suppression of worker fertility in the honey bee (*Apis mellifera*) by treatment with X-rays. Journal of Apicultural Research 45: 27–32.

70. Gao J, Zhao G, Yu Y, Liu F (2010) High Concentration of Nectar Quercetin Enhances Worker Resistance to Queen's Signals in Bees. Journal of Chemical Ecology 36: 1241–1243.

71. Oldroyd BP, Halling L, Rinderer TE (1999) Development and behaviour of anarchistic honeybees. Proceedings of the Royal Society of London B: 1875–1878.

72. Chaud-Netto J, Bueno OC (1979) Number of ovarioles in workers of *Apis mellifera adansonii* and *Apis mellifera ligustica*: A comparative study. Journal of Apicultural Research 18: 260–263.

73. Linksvayer TA, Rueppell O, Siegel A, Kaftanoglu O, Page REJ, et al. (2009) The Genetic Basis of Transgressive Ovary Size in Honeybee Workers. Genetics 183: 693–707.

74. Patel A, Fondrk MK, Kaftanoglu O, Emore C, Hunt G, et al. (2007) The making of a queen: TOR pathway is a key player in diphenic caste development. Public Library of Science ONE 2: e509.

75. Montague CE, Oldroyd BP (1998) The evolution of worker sterility in honey bees: an investigation into a behavioral mutant. Evolution 52: 1408–1415.

76. Page R, Erickson E (1988) Reproduction by worker honey bees (*Apis mellifera*) L.). Behavioural Ecology and Sociobiology 23: 117–126.

77. Winston M, Slessor K (1992) The essence of royalty - honey-bee queen pheromone. American Scientist 80: 374–385.

78. Caspi A, Moffitt TE (2006) Gene-environment interactions in psychiatry: joining forces with neuroscience. Nature Reviews Neuroscience 7: 583–590.

79. Alaux C, Le Conte Y, Adams HA, Rodriguez-Zas SL, Grozinger CM, et al. (2009) Regulation of brain gene expression in honey bees by brood pheromone. Genes, Brain and Behaviour 8: 309–319.

80. Grozinger CM, Fischer P, Hampton JE (2007) Uncoupling primer and releaser responses to pheromone in honey bees. Naturwissenschaften 94: 375–379.

81. Maisonnasse A, Beslay D, Crauser D, Plettner E, Le Conte Y (2010) New insights into honey bee (*Apis mellifera*) pheromone communication. Is the queen mandibular pheromone alone in colony regulation? Frontiers in Zoology 7: 18.

3. Data Chapter 2

Testing genetic hypotheses of reproductive regulation in honey bees

3.1 Introduction

The primary characteristic that separates eusocial insects from their subsocial and solitary counterparts is a strong division of labour into reproductive and non-reproductive castes. This characteristic has enabled eusocial taxa such as ants, bees and termites to capitalize on the efficiencies of task partitioning, such that their colonies appear organized and cooperative [1]. While some aspects of eusocial behaviour are expected to have a genetic basis [2,3], the molecular basis of division of labour and reproductive altruism remains obscure.

Honey bees belong to the order Hymenoptera, and are male-haploid [4]. It is this genomic feature that inflates relatedness between full sisters and may have favoured the spread of so-called 'genes for altruism' via kin selection [5]. The disproportionate number of eusocial taxa within the Hymenoptera and other male-haploid orders suggests that high relatedness among interacting individuals is important to the evolution of social behaviour, and likely remain so even for taxa with advanced eusocial breeding systems [6]. Despite the implied genetic basis to eusociality, few empirical studies have attempted to identify specific genes that regulate the expression of reproductively altruistic traits.

For honey bee societies one obvious altruistic trait is worker sterility. Here, workers can, and typically do, forego their own reproduction to assist with the queen's reproductive output [7]. Worker sterility, when coupled with helping behaviour, is an extreme form of reproductive altruism, and is conditionally expressed as a function of social context. The principal social cue that triggers a worker's functional sterility is the presence of a fecund queen [8]. Genes that regulate the expression of worker traits, including those that regulate the expression of sterility itself, are in effect candidate genes 'for' worker altruism [3].

Recent work has begun to uncover sets of genes involved in queen-worker caste differentiation [9-11], but these screens do not in themselves identify socially responsive genes that regulate reproduction at the individual level. A more directed screen would ideally identify the very loci that effectively render adult workers sterile as a function of social context. In honey bee societies, these genes may function by simply switching ovaries *off* when workers are queenright (in the presence of a queen) or switching them *on* when queenless. However, as there is an age-related division of labour among the worker caste, not all workers are equally likely to become reproductive. It is young, nurse bees that show the highest propensity to activate their ovaries, and even this behaviour is not uniform within a cohort [8].

Because honey bee queens signal their fecundity to worker offspring by pheromonal cues [12], the genes that regulate worker reproduction via ovary activation are likely pheromone responsive. Moreover, because reproductive regulation in honey bee (and

other social insects) colonies almost certainly evolved from pathways similar to those present in solitary insects, we expect genes regulating sterility to be homologous with genes from solitary insects – a central prediction of the so-called 'reproductive groundplan hypothesis' (RGPH) [13].

The RGPH predicts that reproductive divisions in labour among highly social taxa probably involved the decoupling of the ancestral reproductive cycle (i.e., the reproductive and non-reproductive phases) into two parts, with one now expressed in workers and the other in queens [14]. For example, among those genes implicated in the RGPH [15], ovarian activation in both solitary and eusocial insects is correlated with the expression of *Vitellogenin (Vg),* a gene encoding an egg-yolk precursor [16]. Beyond *Vg*, however, it is not known the extent to which genes implicated by the RGPH correlate in their expression with ovary activation among individuals within single colonies. As a consequence, it is uncertain whether a social breeding system could evolve from a solitary groundplan.

Past work investigating the RGPH has suggested that the insulin/insulin-like growth factor signaling (IIS) pathway is important for reproductive decision-making in social insects [10]. As the insulin-IGF pathway is related to metabolism, the work of Evans and Wheeler [17] generally supports this prediction. I therefore predict that metabolic genes are integral to the reproductive 'switch' described above, and may likewise function as genes for reproductive altruism.

Using *Apis mellifera* as a model, I used a synthetic pheromone treatment as queen signal to experimentally generate cohorts of workers with and without activated ovaries. Workers who activate their ovaries in the absence of queen signal are potential egg-layers, and are thus considered reproductively selfish. Workers that suppress their ovaries in response to queen signal are, by contrast, functionally sterile. Using these contrasting social phenotypes. I performed a series of whole-genome microarray experiments that directly compared gene expression profiles between selfish and altruistic females, and I did so at various stages of worker development. Specifically, I analysed the gene expression differences between reproductive and sterile workers, of age cohorts likely to be nurse bees (Day 4 and 6) and bees about to make the nurse-to-forager transition (Day 8 and 10). My goal was to identify candidate genes that effectively switch honey bee ovaries on or off.

In addition to generating candidate sets of genes for worker sterility, I performed a Gene Ontology (GO) and KEGG PATHWAY analysis [18,19]. These comparative analyses help identify the biological processes and pathways that are associated with candidate gene function and, therefore, may be associated with reproductive regulation in honey bee societies.

3.2 METHODS

3.2.1 Pheromone manipulation of ovary activation

All experiments were conducted in the spring of 2010 using bees produced from three colonies reared at the University of Guelph. Each colony contained a healthy queen of standard commercial Buckfast stock that was allowed to mate with several closely related drones (i.e., full siblings) to minimize variation among F1 workers due to paternal effects.

To manipulate levels of ovarian activation among workers, I used plastic PseudoQueens (Contech Enterprises Inc., Victoria Canada) to expose half the workers to a normalized dose of queen mandibular pheromone (QMP+ treatment), while the other half were exposed to equivalent plastic controls that lacked QMP (QMP- treatment). I created these treatment groups by first collecting late-stage pupae from each colony and incubating them overnight at 32°C. The following morning, 30 newly emerged workers from the same colony were placed in an experimental bee cage (20 cm x 15 cm x 1 5 cm). For each of the three colonies, three replicate QMP+ cages were created by including a PseudoQueen and three replicates QMP- cages were created by including a plastic control (total of 18 cages and 540 workers; Figure 3.2.1). Each cage was fed *ad libitum* with a standard mix of royal jelly, honey and water (9:9:2). On days 4, 6, 8 and 10 post-eclosion, one worker from each cage was collected using ultra-soft forceps and immediately flash-frozen in liquid nitrogen for later RNA analysis. On day 14, all remaining bees were euthanized and dissected to determine the level of ovarian development. Thus, the level of ovarian development

in workers collected for genetic analyses was inferred from the dissections of the Day 14 individuals. This method assumes that all workers of the same age are in the same physiological state, and thus, chronology and not physiology was used to group workers for comparative genetic analyses.

Level of ovary activation was scored using the procedure of Hoover *et al.* [20] following dissection of each bee under a stereoscope (Nikon SMZ 15002). I assigned an ovary activation score based on the single most developed ovary, using a 5-point scale. A score of '0' represented completely inactive, vestigial ovaries with undefined ovarioles, while a score of '4' indicated highly developed ovaries with mature eggs. Scores between these endpoints represented intermediate stages of ovary activation (Table 2.2.1, Chapter 2). To avoid scoring bias, a blind scoring approach was used, where experimental treatment of each bee was unknown at the time of dissection.

To examine the effect of pheromone treatment on ovary activation, a generalized linear model was fit to the ovary Score data. In the model I specified Treatment, Colony and Cage as predictor variables and included the interactions between Treatment × Colony and Treatment × Colony × Cage to test the independence of each variable's effect. As the data collected was effectively "count data", a Poisson distribution in ovary scores was produced. Thus, the model was run with a Poisson error distribution using the Design (v2.3) and Car (v2.0.1) packages in **R** statistical software (*http://cran.r-project.org/*; v 2.12.2).

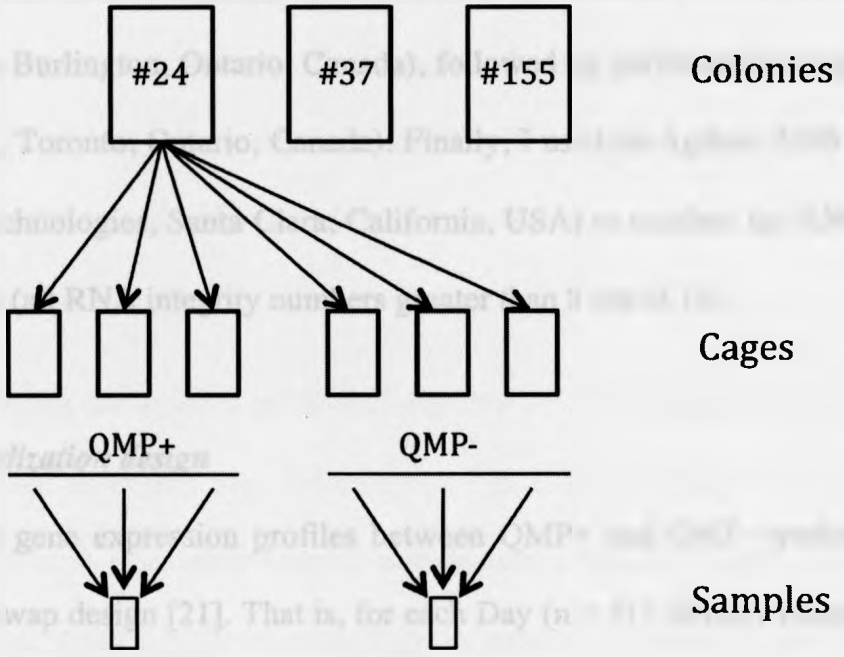


Figure 3.2.1 Schematic diagram of field experimental design and collection of workers for RNA extraction. I used three field colonies (#24, #37, #155) that were each founded by a single queen to seed each cage with n = 30 F1 workers. I used replicate sets of three cages from each colony in QMP+ and QMP- treatments, for a total of 18 cages. To avoid pheromonal contamination between cages, I kept QMP+ and QMP- cages in isolated but environmentally identical incubators.

3.2.2 RNA extraction and quality control

I used a scalpel and fine forceps to carefully remove the exoskeleton, eyes and mandibles from the head of each sampled worker. Insects were placed on dry ice to preserve the brain tissue dissected out and stored at -80°C for < 2 days before RNA extraction. To increase RNA yields, I pooled the brain tissue of three bees from the same colony for each sampling day and treatment. I extracted total RNA using TRIzol (Invitrogen, Burlington, Ontario, Canada), followed by purification using an RNeasy kit (Qiagen, Toronto, Ontario, Canada). Finally, I used an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) to confirm the RNA quality of my samples (all RNA integrity numbers greater than 8 out of 10).

3.2.3 Hybridization design

To contrast gene expression profiles between QMP+ and QMP- workers, I used a direct dye-swap design [21]. That is, for each Day (n = 4) I directly compared QMP+ versus QMP- bees within each colony, and controlled for dye effects by 'swapping' dyes between duplicate samples. Thus, the experiment used two (reciprocally-labeled) arrays to compare the QMP+ and QMP- samples for each colony on each Day (n = 24 arrays).

3.2.4 cDNA synthesis and fluorescent labeling

To prepare RNA samples for microarray analysis, I used the MessageAmp™ Premier RNA Amplification kit (Ambion, Forest City, California, USA) according to manufacturer's protocol. This kit is used to selectively amplify the mRNA, and

results in amplified antisense RNA (aaRNA), which can be hybridized onto an array. I then labeled equal amounts of the aaRNA samples with Cy3 or Cy5 dye using a ULS™ aRNA Labeling kit (Kreatech Diagnostics, Amsterdam, The Netherlands). I divided each pooled aaRNA sample into two equal volumes, and incubated 4 µg of each with either Cy3-ULS or Cy5-ULS and 1X labeling solution at 85°C for 30 minutes. After using the kit-prescribed incubation times and volumes to complete the labeling reactions, I transferred the samples to ice and purified them using the KREA*pure* (Kreatech Diagnostics, Amsterdam, The Netherlands) centrifugal column procedure that is described by the labeling kit protocol.

After calculating the amount of labeled material for each sample, using a NanoDrop (Thermo Fisher Scientific Inc., Wilmington, DE, USA), I combined a recommended 120 pmol of each alternately-labeled sample into a single volume, and dried these down to 9 µL using a vacuum centrifuge to ensure a constant probe concentration between samples. I then fragmented the aaRNA samples using 10X fragmentation buffer (Ambion, Applied Biosystems, Forest City, California, USA), and incubating the samples at 70°C for 15 minutes. Stop solution (included with the Ambion kit) and ice were used to stop the fragmentation reaction, and samples were kept at -80°C until hybridization.

3.2.5 Comparative genomic hybridizations

Whole-genome oligonucleotide arrays (Honey bee oligo 13K v1) were supplied by the laboratory of Dr. Gene Robinson (University of Illinois Urbana-Champaign).

There were 13,440 oligo probes printed onto the array, which were spotted in duplicate. They included all predicted genes from the honey bee genome, as well as ESTs and additional markers for bee parasites and pathogens. The complete technical specifications of the array are described at the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number A-MEXP-755.

To hybridize labeled aaRNA onto individual arrays, I first mixed the samples 2x formamide hybridization buffer and KREAblock solution following the manufacturer's instructions (Kreatech Diagnostics, Amsterdam, The Netherlands). The hybridization solution was then denatured at 80°C for 10 min. Finally, I added 11 µl of the hybridization solution to the surface of the microarray and incubated it for 16 hours at 42°C in an InSlide Out™ hybridization oven (Boekel Scientific, Pennsylvania, USA).

Following incubation, I prepared arrays for scanning by using a series of detergent washes to remove any label that was not bound to the array. All washes were performed in the dark for 11 minutes at 100 rpm on a platform shaker. The first wash consisted of 0.2% SDS and 2% SSC at 42°C, the second wash consisted of 2% SSC at room temperature and the third wash consisted of 0.2% SSC at room temperature. After the final wash, I dried the arrays in a centrifuge for 2 min at 500 g.

3.2.6 Array scanning and data acquisition

I used a VersArray Chip Reader (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) to acquire raw fluorescent data from individual arrays and to produce a pseudocolour TIFF file for the each channel (Cy3 and Cy5). When the Cy3 (green) and Cy5 (red) labeled TIFF files are overlaid, the relative expression of green-red pixel intensity can be estimated and used as an indicator of underlying gene expression [22]. I made red-green colour assignments and performed all image acquisition using ArrayVision (v6.0, GE HealthCare, UK) software. I then used the raw red-green intensity data to calculate the relative expression value of each gene, following a series of quality control, background correction (see 3.2.7), and technical normalization steps.

3.2.7 Background correction and data normalization

I again used ArrayVision software to calculate the mean pixel intensity of each spot. This calculation consisted of two preprocessing steps. First, I removed individual pixels deemed by the software to represent image artifacts (e.g., dust particles). Second, for the remaining pixels, additional non-specific (background) hybridization signal was discounted by subtracting the mean background from the mean foreground pixel intensity values (performed by ArrayVision). All background-subtracted intensities with a value less than '1' were automatically edited to a value of '1' to avoid technical errors upon $\log_2$ transformation.

I further accounted for intensity-dependent and spatial biases in hybridization signal by fitting a lowess (locally weighted) regression to $\log_2$ transformed red/green data. This method is used to smooth the Ratio x Intensity (RI) plots for each print-tip group (n = 48) for each array (n = 24). The residuals from these regressions represent log-ratios that are free of intensity or spatial biases [23]. I therefore used these unbiased (normalised) log-ratios when calculating the biological expression of each gene.

To calculate an expression-fold difference between treated and control samples for each gene I first averaged the normalised expression values between duplicate array spots (n = 2, in each case). I then fit a linear model to the gene-wise data matrix [24] using the MA/ANOVA software package (v1.22.0) [25] within the **R** statistical environment.

3.2.8 Statistical analysis of differential gene expression

I applied a separate ANOVA to each of the four time points in the field study (i.e., Day 4, Day 6, Day 8, Day 10). I therefore used MA/ANOVA to fit a linear model to three pairs of red-green data sets (arrays) per day, as specified by my experimental design. In a first stage of this analysis, only the fixed variable (Treatment) is used to explain variation in the data. MA/ANOVA then adds additional variables (Dye, Array, ArrayBatch) to the model in an effort to improve goodness-of-fit against the observed data. By partitioning variation into fixed and random effects (symbolized by '~' in the model notation below), the program is able to accurately estimate the Treatment effect by controlling for random effects. The full model is specified as follows:

$$Y = \mu + \sim Array + Dye + \sim Colony + \sim ArrayBatch + Treatment + \varepsilon$$

In the model, Y is the log-transformed red-green intensity, which has been decomposed into the sum of the various effects; μ is the overall average intensity of the gene, and ε is the measurement error. I used the model to calculate an F-statistic for each gene, specifically according to Treatment. I followed convention [26] and considered a gene to be differentially expressed when the fixed factor or its interaction term yielded a P-value < 0.001. For each day, I generated a set of differentially expressed genes (DEG) ranked by the probability for differential expression.

To visualize any overlap in DEG sets identified for each Day, I generated a simple four-set Venn diagram using the on-line bioinformatic tool VENNY (*http://bioinfogp.cnb.csic.es/tools /venny/index.html*). I used a chi-squared test to test for directional bias in gene expression patterns (i.e., up- vs. down-regulated) between Days.

3.2.9 Gene ontology and pathway analysis

I analysed my DEG lists for biological meaning using the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7) [27]. I first uploaded each gene list (n=4) into the online Analysis Wizard and assigned *Drosophila melanogaster* as the background reference annotation database. The functional annotation module within DAVID then assigns a GO annotation term to each gene within each of my four DEG lists, for which there was a fly ortholog.

In addition to the gene-by-gene annotation, I tested if particular GO or KEGG annotation terms were enriched within any one of the gene lists. For these enrichment analyses, I again used *D. melanogaster* as a background reference genome and identified enriched terms by applying a hypergeometric test [28] to each of my gene lists, as implemented in WebGestalt software [18]. To guard against Type 1 errors associated with multiple-testing, I applied an FDR correction to the hypergeometic test [29]. For both of these descriptive analyses, I used a significance threshold of $\alpha = 0.05$. This inclusive threshold increases the number of *D. melanogaster* orthologs available for this exploratory analysis.

3.3 RESULTS

3.3.1 Ovarian development

A description of ovary activation scores as revealed from microdissections of individual bees is already described within this thesis. See section 2.3.1 and Figure 2.3.1.

3.3.2 Patterns of gene expression

MA/ANOVA identified 54 genes that were differentially expressed between QMP+ and QMP- workers on at least one sampling day (Figure 3.3.2). Specifically, 12 genes were differentially expressed on Day 4, 22 genes were differentially expressed on Day 6, 9 genes were differentially expressed on Day 8, and 11 genes were differentially expressed on Day 10. From these data, there was no obvious bias in whether genes were up- or down-regulated as a function of treatment contrasts (Table 3.3.1).

Table 3.3.1 The effect of queen mandibular pheromone (QMP) on brain gene expression patterns in honey bee workers at various ages (Days, post eclosion). A chi-squared tested was used to determine whether genes were consistently turned 'off' or 'on' (in this case up- or down-regulated) upon ovarian development.

Day	No. Genes	Up-regulated	Down-regulated	χ^2	d.f.	*P*
4	12	3	9	3.00	1	0.08
6	22	12	10	0.18	1	0.67
8	9	3	6	1.00	1	0.32
10	11	7	4	0.82	1	0.37

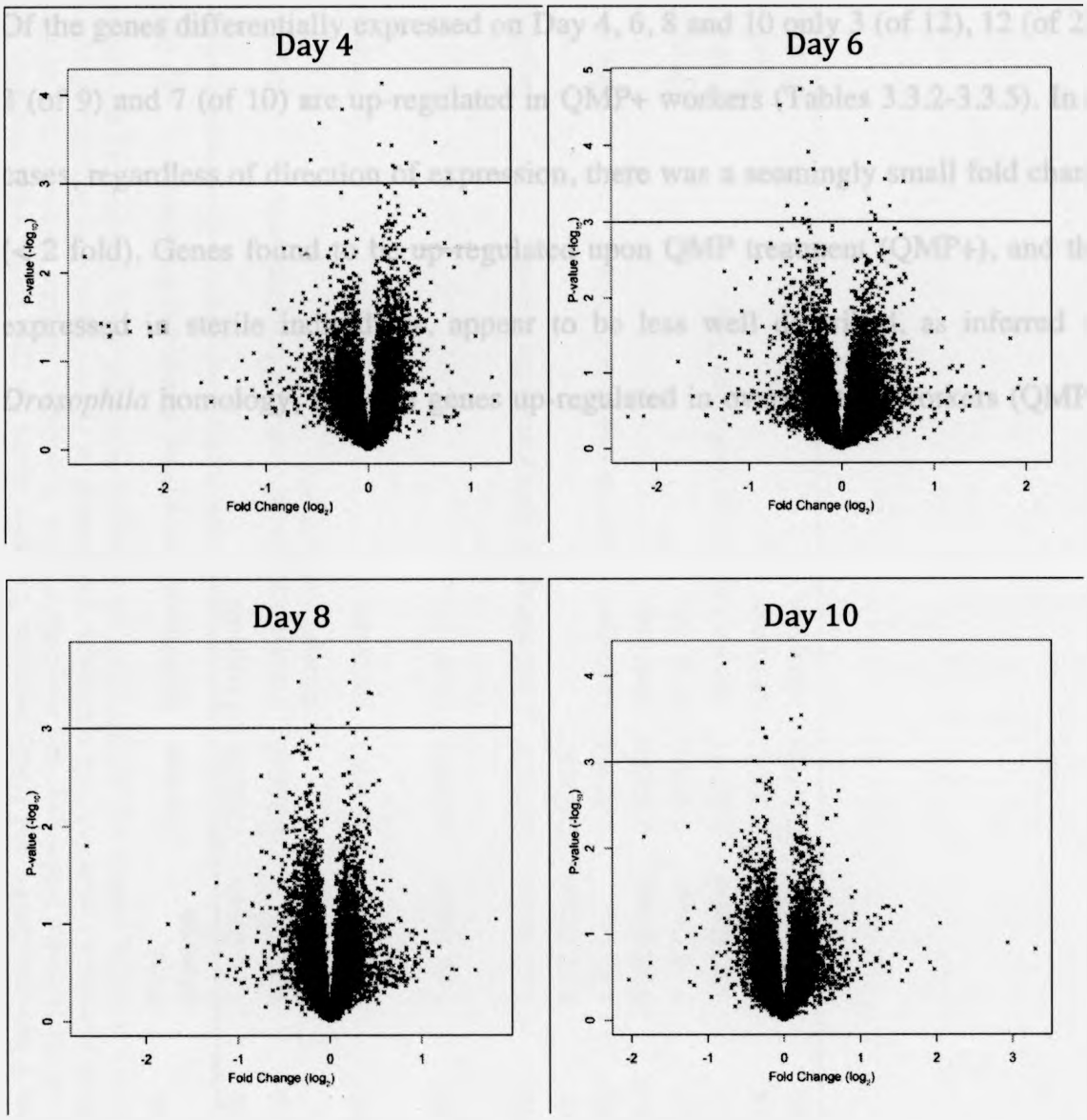


Figure 3.3.2 Effect of queen mandibular pheromone (QMP) on brain gene expression in honey bee workers. Each volcano plot shows the likelihood that the gene is differentially expressed (*P*-value) as a function of its fold-change on that sampling day. Positive fold changes indicate that a gene was up-regulated in workers from cages with high ovary activation (QMP-) relative to workers from cages with low ovary activation (QMP+). The horizontal line represents a significance threshold of $\alpha = 0.001$. Each plot shows n = 13,441 array genes.

3.3.3 Candidate gene identification, by day

Of the genes differentially expressed on Day 4, 6, 8 and 10 only 3 (of 12), 12 (of 22), 3 (of 9) and 7 (of 10) are up-regulated in QMP+ workers (Tables 3.3.2-3.3.5). In all cases, regardless of direction of expression, there was a seemingly small fold change (< 2 fold). Genes found to be up-regulated upon QMP treatment (QMP+), and thus expressed in sterile individuals, appear to be less well described, as inferred via *Drosophila* homology, than the genes up-regulated in ovary active workers (QMP-).

Table 3.3.2 Genes differentially expressed on Day 4 between QMP+ and QMP- workers. The table shows the gene ID or, where available, accession number, the unique oligo identifier from the array, and the estimated fold-change and associated *P*-value. In addition, the table shows the inferred Biological Processes and Molecular Functions, using the fly ortholog (terms are separated with semi-colon, with Biological Process followed by Molecular Function). An up fold change indicates a gene that had higher expression in QMP+ workers than in QMP- workers, whereas a down fold change indicated the opposite pattern.

Gene ID	Arrayset Name	Fold Change	*P*-value	Biological Processes; Molecular Function	Fly Ortholog
AM00836	AM00836	1.10 Down	7.4E-05	Unknown	
Adipocyte acid phosphatase (GB16933)	AM09510	1.18 Up	0.00015	Protein amino acid dephosphorylation; acid phosphatase activity	FBgn0040076
F-box and leucine-rich repeat protein (GB19733)	AM12280	1.38 Up	0.00021	Unknown	FBgn0035959
Odorant binding protein 3 (Obp3) (GB30242)	AM12005	1.58 Down	0.00035	Unknown	
GB17035	AM09612	1.07 Down	0.00037	Lipid transport; lipid transporter activity	FBgn0032136
BI504140	AM01180	1.17 Down	0.00037		
GB30336	AM00328	1.47 Up	0.00054	Unknown; structural molecule activity	FBgn0037069
Unknown	AM02624R	1.29 Down	0.00060	Unknown	
GB18881	AM11438	1.18 Down	0.00064	Unknown	FBgn0032499
GB10732	AM03357	1.20 Down	0.00067	Protein folding; nucleotide binding	FBgn0023529
GB10217	AM02844	1.40 Down	0.00082	Unknown	FBgn0025681
GB12936	AM05548	1.72 Down	0.00088	Unknown	FBgn0052137

Table 3.3.3 Genes differentially expressed on Day 6 between QMP+ and QMP- workers. The table shows the gene ID or, where available, accession number, the unique oligo identifier from the array, and the estimated fold-change and associated *P*-value. In addition, the table shows the inferred Biological Processes and Molecular Functions, using the fly ortholog (terms are separated with semi-colon, with Biological Process followed by Molecular Function). An up fold change indicates a gene that had higher expression in QMP+ workers than in QMP- workers, whereas a down fold change indicated the opposite pattern.

Name	Arrayset Name	Fold Change	P-value	Biological Processes and Molecular Function	Fly Ortholog
GB16994	AM09571	1.24 Up	1.47E-05	Unknown	FBgn0031993
linkage group 4 genomic contig	AM00130	1.35 Up	1.80E-05	Unknown	
Histidine decarboxylase (GB10303)	AM02933	1.58 Up	2.93E-05	Compound eye development; Histidine decarboxylase activity	FBgn0005619
GB19547	AM12099	1.21 Down	4.58E-05	Unknown; monocarbolxylic acid transmembrane transporter activity	FBgn0023549
Fuzzy (GB16647)	AM09225	1.27 Up	0.00012	Cell morphogenesis, establishment of planar polarity; Unknown	FBgn0001084
Dauer Up-Regulated family member (dur-1) (BI512489)	AM02032	1.23 Down	0.00017	Unknown	FBgn0015390
GB20128	AM12675	1.23 Up	0.00021	Unknown	FBgn0030076
GB11880	AM04498	1.38 Down	0.00028	Transcription, speramatogenesis; DNA binding, zinc ion binding	FBgn0037751
Centrosomal protein 190kD (GB17743)	AM10316	1.59 Down	0.00030	Chromatin organization; DNA binding, microtubule binding	FBgn0000283
Spermatogenesis associated 13 (GB13057)	AM05666	1.03 Down	0.00033	Unknown	FBgn0035128
BH10041D13	AM00621	1.10 Up	0.00037	Unknown	
Cyclophilin 1 (GB12544)	AM05160	1.23 Down	0.00050	Protein folding, salivary gland development; peptidyl-prolyl cis-trans isomerase activity	FBgn0004432
Prophenoloxidase (GB18313)	AM10878	1.29 Up	0.00058	Melanin metabolic process; monophenol monooxygenase activity	FBgn0033367
GB17444	AM10016	1.49 Up	0.00061	Unknown	FBgn0038642
GB11507	AM04127	1.43 Down	0.00062	Unknown	

Name	Arrayset Name	Fold Change	P-value	Biological Processes and Molecular Function	Fly Ortholog
vegetable (GB17881)	AM10452	1.25 Down	0.00074	Protein amino acid lipidation; mannosyltransferase activity	FBgn0015562
GB16455	AM09034	1.28 Down	0.00082	DNA catabolic process; Nuclease activity	FBgn0028406
GB17124	AM09699	1.33 Up	0.00090	tRNA metabolic process, translation; nucleotide binding	FBgn0028481
GB11251	AM03874	1.42 Up	0.00090	Oxidation reduction; Nucleotide binding	FBgn0036824
pickpocket 28 (GB30567)	AM12279	1.29 Down	0.00093	Cation transport; metal ion transmembrane transporter activity	FBgn0030795
Na pump subunit (GB10508)	AM03135	1.27 Up	0.00095	Purine nucleotide metabolic process; nucleotide binding	FBgn0002921
Sorting nexin-17 (GB10004)	AM02633	1.29 Up	0.00099	Unknown; Phospholipid binding	FBgn0032191

Table 3.3.4 Genes differentially expressed on Day 8 between QMP+ and QMP- workers. The table shows the gene ID or, where available, accession number, the unique oligo identifier from the array, and the estimated fold-change and associated *P*-value. In addition, the table shows the inferred Biological Processes and Molecular Functions, using the fly ortholog (terms are separated with semi-colon, with Biological Process followed by Molecular Function). An up fold change indicates a gene that had higher expression in QMP+ workers than in QMP- workers, whereas a down fold change indicated the opposite pattern.

Name	Arrayset Name	Fold Change	P-value	Biological Processes and Molecular Function	Fly Ortholog
BI504328	AM01194	1.07 Up	0.00018	Unknown	
Histidyl-tRNA synthetase (GB19522)	AM12074	1.19 Down	0.00020	Translation; nucleotide binding	FBgn0027087
DB779807	AM12841	1.26 Up	0.00033	Unknown	
Probable ATP-dependent RNA helicase kurz (GB12160)	AM04781	1.16 Down	0.00034	Unknown; nucleotide binding	FBgn0001330
Threonyl-tRNA synthetase (GB18377)	AM10940	1.34 Down	0.00043	Unknown; tRNA metabolic process, translation	FBgn0027081
Smrter (GB30103)	AM03245	1.37 Down	0.00044	Regulation of transcription (DNA-dependent); DNA binding	FBgn0024308
traffic jam (GB18094)	AM10664	1.24 Down	0.00064	Reproductive developmental process, transcription; DNA binding, transcription factor activity	FBgn0000964
BI510623	AM01832	1.15 Down	0.00089	Unknown	FBgn0038460
GB12965	AM05578	1.13 Up	0.00094	Unknown; KU70 binding	FBgn0032644

Table 3.3.5 Genes differentially expressed on Day 10 between QMP+ and QMP- workers. The table shows the gene ID or, where available, accession number, the unique oligo identifier from the array, and the estimated fold-change and associated *P*-value. In addition, the table shows the inferred Biological Processes and Molecular Functions, using the fly ortholog (terms are separated with semi-colon, with Biological Process followed by Molecular Function). An up fold change indicates a gene that had higher expression in QMP+ workers than in QMP- workers, whereas a down fold change indicated the opposite pattern.

Name	Arrayset Name	Fold Change	P-value	Biological Processes and Molecular Function	Fly Ortholog
GB30266	AM12715	1.08 Down	5.76E-05	Unknown	FBgn0053170
BI514760	AM02314	1.21 Up	7.03E-05	Unknown	
GB30530	AM06996	1.70 Up	7.20E-05	Unknown; 1-acylglycerol-3-phosphate O-acyltransferase activity	FBgn0034971
GB19806	AM12353	1.20 Up	0.00014	Unknown	FBgn0050118
Tetraspanin 66E (GB13255)	AM12795	1.17 Down	0.00029	Unknown	FBgn0035936
Putative transcription factor mblk-1 (BI516137)	AM02500	1.07 Down	0.00032	Instar larval or pupal development, regulation of transcription (DNA-dependent); DNA binding, transcription factor activity	FBgn0013948
GB30023	AM12734	1.15 Down	0.00040	Unknown	
GB30410	AM06979	1.20 Up	0.00041	Mitotic sister chromatid separation; nucleotide binding	FBgn0015391
GB15669	AM08252	1.18 Up	0.00052	Unknown	FBgn0030205
GB17013	AM09589	1.16 Up	0.00053	Amino acid transport; amine transmembrane transporter activity	FBgn0036116
GB10854	AM03479	1.30 Up	0.0010	Unknown; carboxylesterase activity	FBgn0015575

3.3.4 Candidate gene identification, across multiple days

There was no overlap in the genes that were differentially expressed between days when I used a stringent significance cutoff of $P < 0.001$ for the Venn analysis. That is, all of the differentially expressed genes identified by the previous analyses were specific to a single time-point. However, when I used a more inclusive significance cutoff of $P < 0.05$ (encompassing 564, 782, 623 and 534 genes on Days 4, 6, 8 and 10), I identified a number of genes that had similar expression patterns across consecutive days (Table A.1, Appendix). Specifically, in young bees (days 4 and 6), there were 4 genes that had higher expression and 9 genes that had lower expression in QMP+ workers relative to QMP- workers. In intermediate-aged bees (days 6 and 8), there were 11 genes that had higher expression and 4 genes that had lower expression in QMP+ workers relative to QMP- workers. In older bees (days 8 and 10), there were 13 genes that had higher expression and 5 genes that had lower expression in QMP+ workers relative to QMP- workers. Thus, in general, it was found that gene expression was seldom consistent, and changed with age. However, I found a single gene that was differentially expressed across all four days: GB15506 was consistently up-regulated in QMP+ workers relative to QMP- workers.

Functions can be inferred for a number of the genes that showed consistent differences in expression across days. Four of the genes that were differentially expressed on Day 4 and 6 are involved in nucleotide binding: F-box and leucine-rich repeat protein 3 (*Fbxl3*, GB19733) and GB13619 (fly Pyridoxine 5-phosphate oxidase) were both up-regulated in QMP+ bees, while GB14629 and GB19480 were

down-regulated in QMP+ bees. Two genes predicted to be involved in cell morphogenesis, the QMP+ down-regulated GB12005 (fly *Myospheroid*) and the up-regulated GB14145 (*Anarchy 1*) were also differentially expressed on Day 4 and 6. GB13606, which has an unknown function but has been identified as a candidate gene for behavioural maturation in honey bees [30], was the last gene down-regulated in QMP+ workers on Day 4 and 6. On Day 6 and 8, major royal jelly protein (MRJP) related GB19132 (*yellow-d*), the neurosparsin queen brain-selective protein-1 (*Qbp-1*), and putative transcription factor GB16549 (fly homeobox only domain) were found to be up-regulated in QMP+ workers. There were two metabolic genes that differed in expression on day 6 and 8: GB13140 (up-regulated in QMP+ workers) and GB17093 (down-regulated in QMP+ workers). On Day 6 and 8, three genes involved in metabolism were up-regulated in QMP+ workers: GB14677, GB13199, and GB16636 (fly *bola-like 3*). Similarly, GB15664 (fly *karst*), involved in gamete generation, and GB19232, involved in mushroom body development and oxidation-reduction were also up-regulated in QMP+ workers. On Day 8 and 10, the metabolic genes GB17267 (fly enhancer of rudimentary), GB16827 (fly *black*), and GB15677 (fly prolyl-4-hydroxylase) were down-regulated in QMP+ workers. On day 4, 6, and 8, GB12375 (fly *Chd64*) was up-regulated in QMP+ workers. Finally, the only gene that differed between treatments in expression on all four days was GB15506, which is likely involved in oxidation-reduction and was always up-regulated in QMP+ workers.

3.3.5 Functional analysis of candidate genes

I identified a total of 1,311 orthologous gene pairs between *A. mellifera* and *D. melanogaster*, which represents about 52.2% of the genes from the inclusive gene lists ($P < 0.05$) that had an associated fly counterpart. These orthologs were used to perform the functional term enrichment and pathways analysis through WebGestalt.

Across all days and treatments, a GO analysis showed that most of the candidate genes that differed in expression between QMP+ and QMP- workers were associated with 'Metabolic process' (the transformation of chemical substances; GO:0008152), 'Localization' (the transportation and maintenance of a substance to a specified location; GO:0051179), and 'Developmental process' (the progression of an organism over time; GO:0032502). The relative proportions of genes associated with each of these processes across days and treatments were very similar, with 'Metabolic process' consistently representing the largest proportion of genes that differed in expression levels (Fig 3.3.3).

I further analyzed the patterns identified by the GO analysis by breaking down the categories into a number of more specific subcategories. Specifically, there were enriched terms associated with 'Metabolic process' in the QMP+ lists on Day 4, 8 and 10, and in the QMP- lists on Day 6, 8 and 10. 'Localization' had enriched terms associated with it in the Day 10 QMP+ list, and the Day 4, 6 and 10 QMP- lists. 'Developmental process' had associated enriched terms in the Day 6, 8 and 10 QMP+ lists, and Day 10 QMP- list. As the GO analysis suggests, genes involved in nitrogen

compound and nucleic acid metabolism appear to be important in sterile (QMP+) workers, while carbohydrate metabolism is important to older, reproductive (QMP-) workers (Table 3.3.5). The importance of genes involved in localization only appears to be so for young, reproductive (QMP-) workers (Table 3.3.6), while developmental differentially expressed genes appear to be slightly biased towards younger, sterile (QMP+) workers (Table 3.3.7). Complete lists and hierarchical diagrams are provided in the Appendix (Fig A1-A8, Appendix).

For the KEGG PATHWAY, each list only had one associated enriched pathway. Specifically, "DNA replication" (4 genes, P = 0.0008), "Metabolic pathways" (17 genes, P = 0.0085), "Metabolic pathways" (24 genes, P = 7.46e-6) and "Metabolic pathways" (24 genes, P = 5.56e-5) were enriched on Day 4, 6, 8 and 10 in QMP+ workers. While "Metabolic pathways" (23 genes, P = 0.0002), "Proteasome" (4 genes, P = 0.0056), "Metabolic pathways" (21 genes, P = 2.12e-5) and "RNA degradation" (4 genes, P = 0.048) were enriched on Day 4, 6, 8 and 10 in QMP- workers.

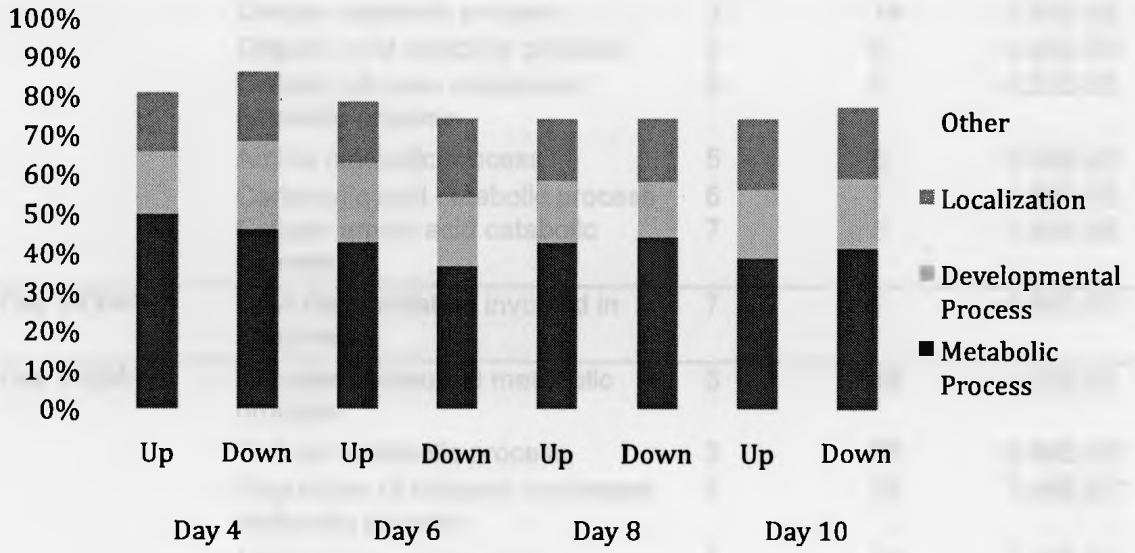


Figure 3.3.3 Proportion of candidate genes ($P < 0.05$) associated with 'Metabolic process', 'Developmental process', and 'Localization' as identified by WebGestalt. "Up" and "Down" refer to the groups of genes up- and down-regulated in the sterile (QMP+) workers.

Table 3.3.6 Enriched GO terms associated with the broad functional term 'Metabolic process.' Each term listed is a child term to 'Metabolic process,' and the overall relationship is illustrated by the associated term's level in the hierarchy (i.e., how many terms are between 'Metabolic process' and the highlighted term). The number of genes associated with each term, as well as the FDR significance value is displayed. *P*-values with an asterisk are associated with terms within the Top 10 for each Day, but whose significance is below threshold.

Gene List	GO Term	Hierarchy Level	No. of Genes	*P*
Day 4 QMP+	Cellular metabolic process	2	60	1.30E-03
	Cellular macromolecule metabolic process	3	50	1.30E-03
	Cellular catabolic process	3	14	1.30E-03
	Organic acid catabolic process	4	5	4.80E-03
	Cellular nitrogen compound catabolic process	4	5	4.20E-03
	Amine catabolic process	5	5	2.50E-03
	Carboxylic acid catabolic process	6	5	4.80E-03
	Cellular amino acid catabolic process	7	5	1.90E-03
Day 6 QMP-	DNA fragmentation involved in apoptosis	7	2	4.46E-02
Day 8 QMP+	Nitrogen compound metabolic process	3	48	1.13E-03
	Cellular metabolic process	3	67	5.59E-02*
	Regulation of nitrogen compound metabolic process	4	22	7.89E-02*
	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4	41	4.10E-03
	Heterocycle metabolic process	4	10	7.89E-02*
	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5	22	7.89E-02*
	Nucleobase, nucleosid and nucleotide metabolic process	5	10	5.59E-02*
	RNA metabolic process	5	26	7.89E-02*
Day 8 QMP-	Cellular metabolic process	3	60	8.13E-02*
	Amine metabolic process	3	11	7.97E-02*
	Carbohydrate metabolic process	3	14	5.97E-02*
	Carbohydrate biosynthetic process	4	5	5.97E-02*
	Cellular carbohydrate metabolic process	4	9	5.97E-02*
	Glycoprotein metabolic process	5	5	7.97E-02*
	Cellular polysaccharide metabolic process	5	3	7.97E-02*
	Glycoprotein biosynthetic process	6	5	7.97E-02*
Day 10 QMP-	Macromolecule modification	4	19	1.07E-02
	Protein modification process	5	18	1.23E-02

Table 3.3.7 Enriched GO terms associated with the broad functional term 'Localization.' Each term listed is a child term to 'Localization,' and the overall relationship is illustrated by the associated term's level in the hierarchy (i.e., how many terms are between 'Localization' and the highlighted term). The number of genes associated with each term, as well as the FDR significance value is displayed. *P*-values with an asterisk are associated with terms within the Top 10 for each Day, but whose significance is below threshold.

Gene List	GO Term	Hierarchy Level	No. of Genes	*P*
Day 4 QMP-	Regulation of protein localization	4	5	1.18E-02
	Regulation of intracellular transport	5	5	1.18E-02
	Regulation of protein transport	6	5	1.18E-02
	Positive regulation of ion transmembrane transporter activity	8	2	1.18E-02
	Positive regulation of calcium ion transport via store-operated calcium channel activity	11	2	1.18E-02
Day 6 QMP-	Cellular macromolecule localization	3	12	4.46E-02
	Cellular protein localization	4	11	2.86E-02
	Vacuolar transport	5	4	1.91E-02
	Lysosomal transport	6	3	5.16E-02*
	Intracellular protein transport	6	11	1.91E-02
Day 10 QMP-	Vesicle-mediated transport	4	13	1.23E-02

Table 3.3.8 Enriched GO terms associated with the broad functional term 'Developmental process.' Each term listed is a child term to 'Developmental process,' and the overall relationship is illustrated by the associated term's level in the hierarchy (i.e., how many terms are between 'Localization' and the highlighted term). The number of genes associated with each term, as well as the FDR significance value is displayed. *P*-values with an asterisk are associated with terms within the Top 10 for each Day, but whose significance is below threshold.

Gene List	GO Term	Hierarchy Level	No. of Genes	*P*
Day 6 QMP+	Post-embryonic morphogenesis	4	12	7.40E-03
	Instar larval or pupal development	4	22	7.40E-03
	Metamorphosis	5	21	7.40E-03
	Instar larval or pupal morphogenesis	5	21	7.40E-03
	Hair cell differentiation	9	6	7.40E-03
	Non-sensory hair organization	10	6	7.40E-03
	Imaginal disc-derived wing hair organization	11	6	7.40E-03
Day 8 QMP+	Segment specification	6	5	4.66E-02
Day 10 QMP-	Aging	3	7	9.30E-03
	Regulation of pigmentation during development	4	3	1.23E-02
	Multicellular organismal aging	4	7	9.30E-03
	Negative regulation of pigmentation during development	5	2	1.91E-02
	Determination of adult lifespan	5	7	9.30E-03

3.4 Discussion

I have identified 54 genes that were differentially expressed between young QMP+ and QMP- workers that in whole or in part may mediate the reproductive divisions in labour. Of the genes identified, most were involved in physiological processes associated with metabolism, particularly in young, sterile (QMP+) bees, localization in young reproductive (QMP-) bees, and development and morphogenesis. Notable among the differentially expressed genes were histidine decarboxylase (GB10303), *Smrter* (GB30103), and *traffic jam* (GB18094). Alcohol dehydrogenase (GB15506) was also strongly implicated for a role in ovary activation and reproductive decision-making because it was consistently over-expressed QMP+ treated worker brains (across all four sampling days).

3.4.1 Ovarian development

In normal queenright colonies, the environmental cue that maintains inactive ovaries in worker bees is the presence of a healthy, egg-laying queen, as signaled by queen pheromone [12]. Consequently, the presence of QMP within a cage can mimic the presence of a live queen, and has been shown to suppress ovary activation in groups of workers [31]. The pheromonal cue used in my present study is thus effective at generating groups of ovary active and inactive workers that were analogous to reproductively selfish and reproductively altruistic workers within natural colonies. Overall, QMP has a strong and consistent effect on worker ovary activation that was comparable in magnitude to other studies [11,32], and was thus effective in producing

reproductively active and inactive workers for my subsequent gene expression analyses.

3.4.2 Patterns of gene expression

For any given Day, there was a general symmetry in the number of genes that were up- and down-regulated between QMP+ and QMP- workers. This pattern is consistent with previous studies that examined gene expression as a function of queen pheromone [11], and more generally suggests that worker reproduction is not regulated by turning large numbers of genes on or off, but rather by a more subtle mechanism whereby small sets of key genes are either activated or inactivated by the effects of social cues. This observation is at odds with the 'gene on to turn off reproduction' hypothesis of Thompson *et al.* [33] which suggested that a greater proportion of genes need to be turned *on* to turn *off* reproduction in workers (i.e. that the effects of many genes are needed to maintain workers in the altruistic helper state). Instead, the symmetric pattern observed here supports the null expectation that similar numbers of genes are needed in either reproductive state. Interestingly, the genes that were up-regulated in QMP+ (sterile) workers appear to be relatively recently derived in honey bees, as no homologs were identified in *Drosophila*. This pattern is consistent with the findings of Barchuk *et al.* [9], who suggests that worker physiology and behaviour arise from the effects of novel genes that have evolved in the order Hymenoptera.

Work on primitively social insects, such as paper wasps (*Polistes metricus*) has also recently begun. The level of sociality in *Polistes* varies greatly between species, and almost all conceivable stages in the evolution of caste can be observed [34]. Across all species, the worker caste is kept in a subordinate position by acts of aggressive dominance from the reigning egg-laying females, who show no external morphological differences from the sterile caste. In a colony, the degree of ovarian development is strongly correlated with hierarchical position [34]. Toth *et al.* [35] compared the brain gene expression of groups of reproductive and sterile paper wasps (*Polistes metricus*), which are primitively eusocial (the reproductive castes are not morphologically distinct), to those of reproductive and sterile honey bees. They found that the overlap between differentially expressed genes between the two behavioural groups had non-significant overlap between the two species. Unlike honey bees, paper wasp ecdysteriod titre remains coupled with reproductive function, and thus the genetic mechanisms regulating egg-laying behaviour in these wasps may more closely resemble those of solitary ancestors [35]. Although these species are related, this disparate result is still consistent with other findings. For example, Weil *et al.* [36] found that only 3 out of 10 genes associated with queen-worker caste differences showed conserved expression patterns across two closely related species of *Cryptocercus* termites. As *Polistes* are more ancestral in their hormone patterns as they relate to reproductive behaviour, they represent an intermediate between solitary insects, such as *Drosophila*, and advanced eusocial insects, such as honey bees. Thus, it is possible that sterility in social Hymenoptera is due to lineage-specific evolutionary events, resulting in honey bee castes and workers with a novel suite of

genes that inhibit ovarian development through complex in-hive chemical signals. Conversely, paper wasps, may have a less derived system, which makes them more similar to solitary ancestors, and likely *Drosophila*, in their genomic expression as it relates to reproductive behaviour. This is supported by the absence of morphologically-distinct castes in wasps, and aggression-based reproductive hierarchy.

3.4.3 Candidate gene identification

Twelve genes were differentially expressed on Day 4, however, only 3 of those 12 genes were identifiable through sequence homology with *D. melanogaster*: Adipocyte acid phosphatatse, F-box and leucine-rich repeat protein (*Fbx13*), and odorant binding protein 3 (*Obp3*). *Fbx13* (GB19733) is apparently a 'honey bee-vertebrate-sequence' that is shared between vertebrates and honey bees, but is absent in all other sequenced insect and nematode genomes [37]. I found that *Fbx13* was up-regulated (1.38-fold) in the brains of QMP+ (sterile) workers, consistent with previous findings that this gene is expressed in the brains of queenright bees [37]. The function of the gene is currently unknown, however, the gene is a likely candidate for worker sterility because of its potential function as an aid in protein-protein interactions, and its presence on two gene lists in my study (it is also marginally up-regulated on Day 6, 1.43-fold, $P < 0.05$). The phosphatase enzyme identified from the Day 4 screen (GB16833) may also be of importance because of its ability to activate or deactivate other enzymes or enable protein-protein interactions. It has been found that phosphatases are integral to many signal transduction pathways [38].

Eleven of 12 genes identified as differentially expressed on Day 6 are identifiable through sequence homology with *D. melanogaster*. Notably, histidine decarboxylase (HDC; GB10303) produces histamine, a biogenic amine that functions as an important neurotransmitter, from histidine [39,40]. Histamine is synthesized in the vertebrate central nervous system in a small population of neurons located in the posterior hypothalamus, which have been implicated in hormonal secretion, cardiovascular control, thermoregulation, and memory [41]. Because of the implied hormonal regulation of reproduction within social insects, it is possible that HDC plays a role in regulating this major neurotransmitter. More interesting still, is that histimine and dopamine are antagonistic [42], as when one is high the other is low. It has been found that reproductive workers have higher dopamine levels than queenright sterile workers [43,44].

By controlling temperature and day-length researchers have been able to manipulate reproductive development in *Drosophila* by forcing females to enter a state of diapause, or a period of developmental arrest [45]. This ability is best known to allow insects to circumvent adverse weather by entering a diapause-reproduction cycle. Diapause states are also common in other arthropods and invertebrates. For example, *Caenorhabditis elegans* can enter a dauer larval stage where the nematode does not feed or grow. However, this state is induced by starvation and overcrowding [46]. The presence of dauer up-regulated family member (BI512489) in Day 6 reproductive (QMP-) individuals appears odd, as a dauer state is correlated with low ecdysteroids

[47]. In this case, dauer up-regulated family member expression was correlated with ecdysteriod levels (as inferred by the reproductive state of the individuals).

SMRT-related ecdysone receptor-interacting factor (*Smrter*; GB30103) is a corepressor that has been described in *Drosophila* as mediating transcription by silencing the ecdysone (an ecdysteriod) receptor-ultraspiracle (EcR:USP) heterodimer [48]. It shows only limited homology to the vertebrate corepressors SMRT and N-CoR. Nevertheless, Tsai *et al.* [48] found that the basic mechanism of repression has been conserved between vertebrates and *Drosophila*. Given this, it is likely that *Smrter* has retained this function in honey bees as ecdysone can function as a gondaotrophic hormone and is associated with oocyte maturation. Thus, it is likely involved in the regulation of genes involved in reproduction and ovarian development, as it was found to be down-regulated in QMP+ individuals on Day 8.

Traffic jam (*tj*; GB18094) has been identified as a transcription factor that is expressed in the somatic gonadal cells during gonadogenesis and gametogensis in *Drosophila* [49]. Male and female flies with *tj* mutations are sterile, as they appear to have severe gonadal defects, but appear normal otherwise. It has been suggested that *tj* may have a specific function in gonadal development, as female flies mutant for the gene had small, disorganized ovaries that lacked mature germ cells. Futher, these females seemed to be missing follicle cells within the ovaries, which is not likely due to a defective germline, but due to the loss of function of *tj* function specifically [49]. Thus, the lack of production of functional germ cells it due to incomplete germ cell

differentiation. As *tj* has only been described in the reproductive tissues, it is not clear how the expression of this gene functions in other tissues, such as the brain (in this case in Day 8 reproductive (QMP-) workers). However, the direction of expression is consistent with what has been found in the literature. Whether *tj* expression is prevalent throughout the worker body, and thus also up-regulated in the ovaries, cannot be determined at this time from the data collected.

Based on the life cycle of the honey bee, I predict that genes that differ in expression between treatments on days 4 and 6 are candidates for initiating differences in maturation, whereas genes that differ in expression on days 8 and 10 likely represent the down-stream result of reproductive maturation. These later genes are likely only affiliated with reproductive behaviour and oocyte maturation, rather than the basis of it. Further, there are very distinct physiological changes that occur within a bee that has transitioned from nurse-to-forager. For instance, the JH titre within a forager increases to relatively high levels, and is correlated with a drop in *Vg* [50]. JH also mediated other life-history traits in workers, including fecundity and lifespan [51,52]. As various cohorts of workers were compared, and very little consistency in gene expression differences was seen, this is likely due to both the age-related physiological changes that take place in worker division of labour as well as the unequal ability of all workers to respond to the same degree to social cues. However, all workers analysed would still be performing in-hive tasks under normal conditions. Thus, if the inconsistency in expression between days is not due to a major age-related change in physiology, then it is likely due to the inability for all workers to

display an equal degree of physiological change to a social cue (in this case, queen pheromone). However, there was a small degree of overlap between age cohorts.

Of the 1,206 genes up-regulated on Day 4, 6, 8, and 10 in my inclusive gene lists ($P < 0.05$), only one gene showed a consistent difference in gene expression between QMP+ and QMP- workers across all days. GB15506, a putitive alcohol dehydrogenase (ADH, oxidoreductase activity) always had higher expression in QMP+ workers than in QMP- workers. *Drosophila* short-chain ADH is one of the molecularly best-characterized enzymes in insects. It is controlled by two promotors, and generates two transcripts of varying size during development [53]. Its expression it thought to be controlled, or at least partially regulated by the expression level of ecdysteroid transcripts [54]. The expression of the *Drosophila* short-chain ADH *Apis* ortholog has been studied by Guidugli *et al.* [55]. It was found that honey bee larvae in the 5th (and final) instar have an ovarian transcription profile that was very similar to that of the adult fly. It has been suggested that the regulation of genes coding for redox reaction enzymes may be a decisive initial step in the differentiation of the caste phenotypes [17,55,56].

Previous studies of honey bees have identified *Anarchy 1* as a prime candidate gene for controlling the reproductive behaviour of workers [57]. Indeed, it was so named due to its expression in a selectively bred anarchist strain of honey bees in which workers frequently display egg-laying behaviour despite the presence of a queen [58,59]. As would be predicted, this gene was up-regulated in reproductively active

(QMP-) workers on Day 4 and 6. The absence of this gene in the Day 8 and 10 gene lists makes the expression of this gene inconsistent in fecund workers. However, this gene may be important early on in the ovarian developmental cascade in wildtype workers, where it may be ubiquitously expressed in the anarchist mutant strain.

The differential expression of major royal jelly protein MRJP-related *yellow-d* (GB19132) is likely symptomatic of a fundamental difference in the reproductive development of the two treatment groups. MRJPs are known to mediate reproductive maturation and the expression of honey bee social behaviour at several levels, and are thought to be co-evolved with *Apis* eusociality, implying an inherent association with the behavioural and biological functions of the MRJPs [60,61]. Their relation to worker reproductive status has been implied in the past [33] and their uniform expression on both Day 6 and 8 in sterile (QMP+) workers is not surprising.

Up-regulated (QMP+) on Day 6 and 8 is aldehyde dehydrogenase type III (GB12681), which is important for carbohydrate metabolism and energy production. This gene has previously been implicated in caste differentiation. It is differentially expressed in worker larvae, relative to queen larvae, 72 hrs after hatching, but eventually reverses its expression pattern after 120 hrs [62]. As it is involved in the glycolysis pathway, it was suggested that this gene is caste-specific and allows queen-destined larvae to follow a different developmental trajectory than worker-destined larvae [62]. This finding, however, suggests that aldehyde dehydrogenase may not be caste-specific, yet may still play a role in caste differentiation, as this study found

aldehyde dehydrogenase to be up-regulated in sterile (QMP+) workers, while Li *et al.* [62] considered it to be queen-biased. However, this may be due to the different physiologies of adult queens and workers as compared to queen- and worker-destined larvae.

Chd64 (GB12375) is JH induced in *D. melanogaster*, and is known to be involved in both JH and ecdysteroid function, suggesting that this protein may play an important role in cross-talk between JH and ecdysteroids [63]. As it has been found in sterile workers on Day 4, 6, and 8, it likely inhibits ovaries of QR workers.

3.4.4 Candidate genes viewed in the context of known reproductive regulatory pathways

In long-lived insects that undergo many cycles of reproduction, vitellogenesis occurs when the fat body is activated by hormones that allow vitellogenin to be produced cyclically. Both juvenile hormone (JH) and ecdysteroids are involved in this process, with JH regulating the formation of new endoplasmic reticulum in the fat body to sequester *Vg* [64] and ecdysteroids regulating the rate of *Vg* production [65]. As a result, oogenesis, which is dependent on vitellogenin production and mobilization from the fat body, is regulated by the two major hormone groups found in insects. Thus, the endocrine system controls the reproductive status of a female insect, and the genes or gene networks regulating the endocrine system serve as the up-stream 'switch.'

Insects have two endocrine glands: the prothoracic glands, which produce ecdysteroids, and the corpus allatum, which produces JH [65,66]. Further, like vertebrates, insects also have nerve cells that generate electrical impulses, which can be translated into chemical messages at the synapse. The chemical messengers, or neurotransmitters (e.g., dopamine, serotonin), that are released can activate an adjacent neuron, or may stimulate endocrine tissues directly [67]. Histidine decarboxylase, a gene that was found to be differentially expressed in the brains of 6-day-old workers (up-regulated in sterile workers), mediates the production of histamine [39], and is possibly a part of the cascade that keeps ovaries inhibited. The insect brain also contains neurosecretory cells, which are specialized neurons that produce chemical messengers (neurohormones) that are released into circulation, in response to environmental stimuli, and affect distant tissues [68]. When neurohormones reach target tissues they initiate the synthesis or release of secondary messenger molecules that carry the message into the cell. This then initiates a cascade of phosphorylations, which alters gene expression patterns within the tissue. The mode of action of neurohormones that stimulate ecdysteroid production in the prothoracic gland has been studied and it is presumed that the phosphorylation of the S6 ribosomal protein causes a change in the translation of certain mRNAs [69,70]. While JH and ecdysteroids are common to all insects, neurohormones are more species-specific [67]. Two prothoracic-stimulating neurohormones have been identified in *Manduca sexta*; β-tubulin and hsp70 [71]. In *Bombyx mori* a prothoracic-stimulating-like neurohormone, bombyxin, has been studied extensively and shows 40% similarity in its primary sequence to human insulin [72]. Although bombyxin

does not affect the production of ecdysteroids, it does have receptors present in the ovaries of some lepidopterans and the hormone may be involved in ovarian development [73].

Although ecdysteroids are primarily involved in the molting process of arthropods, they are known to demonstrate a while range of effects throughout every developmental stage, including the regulation of reproduction. Within a target cell, these hormones bind to an ecdysteroid receptor, which is a heterodimer consisting of EcR and USP [74]. This protein complex acts as a DNA binding protein and nuclear receptor, and the nuclear response is dependent on the EcR isoform. *Smrter*, which was identified as being differentially expressed in day 8 reproductive workers silences the EcR: USP complex in *Drosophila* [48]. JH is similar to ecdysteroids in that it has multiple effects during the life of an adult insect, including metamorphosis, diapause, reproduction and metabolism. The production of this hormone is regulated by environmental stimuli, specifically, mating and nutritional state. These cues are interpreted by the brain and corpora allatum activity is regulated both neurally and by neurosecretory hormones. JH production may also be regulated by ecdysteroids, as ecdysteroid receptors have been found on the corpora allatum of *Manduca*, and in the presence of ecdysteroids JH preserves its current gene expression program [68]. Likewise, the gene product of *Chd64* may mediate the cross-talk between JH and ecdysteroids, as suggested by Li *et al.* [63]. This gene was found to be differentially expressed on day 4, 6, and 8 and was up-regulated in sterile individuals. However, despite the extensive work concerning JH in caste differentiation in honey bees, there

is still little known about the link between embryological markers and JH titre [75,76]. This is likely due to the fact that JH appear to be uncoupled from ovarian development and egg-laying behaviour in honey bees [52]. This JH, along with ecdysteroid, decoupling has also been described in the advanced eusocial stingless bee (*Melipona quandrifasciata*), where it has been suggested that these gonadotropic hormones have lost most of their reproductive functions, yet have gained functions in larval caste development [77].

While insect reproduction is comprised of a succession of interdependent steps, from sex determination to oviposition, all of which are regulated by ecdysteroids, JH and neurohormones, vertebrates, by contrast, utilize gonadotropins, which are all structurally quite homogeneous [67]. Gonadotrophin-releasing hormone (GnRH) is the final signaling molecule secreted from the brain to regulate reproduction in all vertebrates [78]. It is synthesized by neurosecretory cells in the hypothalamus and is transported to the pituitary gland where it stimulates the secretion of lutenizing hormone and follicle-stimulating hormone [78]. It appears that only environmental stimuli and a few internal signals control the release of GnRH. The main internal signal is estrogen, which works through negative feedback as it is released from the ovaries and inhibits GnRH secretion [79]. Thus, both insect and vertebrate reproductive regulation is the result of secretions from the CNS that stimulate the endocrine system. These secretions communicate with the gonads and may stimulate oocyte development.

The genetic screens performed were on the brains of workers only, and as such, may identify brain transcripts involved in ovarian activation, as opposed to downstream transcripts expressed in the actual ovarian tissues. In theory, the assay should have identified expression changes in genes encoding neurosecretory hormones, neurotransmitters, and other enzymes that regulate these two groups. As described in detail above, some of the highly differentially expressed ($P < 0.001$) transcripts as well as the differentially expressed ($P < 0.05$) transcripts identified across at least two consecutive days are likely involved in CNS control of reproduction. Specifically, histidine decarboxylase, Smrter, alcohol dehydrogenase and Chd64 are good candidates in this regard. Further, Fbx13 and Anarchy1 have been identified in the past to be involved in reproductive status (as discussed above), which makes them good candidates for reproductive behaviour as well.

3.4.5 Functional analysis of candidate genes

Overall, I found that candidate genes that differed between treatments were associated with 'Metabolic process', 'Developmental process', and 'Localization.' Expression patterns for these biological processes have also been explored in queen and worker larvae. As determined by Barchuk *et al.* [9], expression patterns between larvae castes appear to be similar to those of the reproductive and sterile workers produced in this study, in that the percentage of metabolic gene expression was relatively consistent over time. Further, the *number* of genes associated with localization was similar between the 'sterile' and 'reproductive' groups. However, during the larvae stages,

developmental genes represented a much larger proportion of the genes expressed in the workers, which varies from the findings on adults of this study.

The frequent association of enriched terms with 'Metabolic process' suggests that processes such as anabolism or catabolism are important to the regulation of reproduction in this insect. These processes can include macromolecular processes such as DNA repair and replication, and protein synthesis and degradation. Indeed, a range of enriched metabolic processes were identified, however, the types of processes differed according to treatment and age. I determined that there was an over-representation of genes involved in nitrogen compound metabolism in sterile (QMP+) workers, specifically on Day 4 and 8. This, in particular is interesting because amino acids are organic nitrogen compounds. If translational levels in certain cell types in the brain are changing, than it could explain the over-representation of genes involved in this nitrogen compound metabolism. Conversely, in reproductive (QMP-) workers, genes involved in carbohydrate metabolism and biosynthesis were over-represented. The hormonal control of caste polyphenism, and thus reproductive altruism, may target energy metabolism because in most cases the initial trigger in insects is a nutritional signal [80]. Thus, hormonal regulation of metabolic enzymes could be interpreted as a feedback loop within caste development [55]. Energy metabolism has been addressed in previous studies on honey bee caste development. It was determined that, as a response to the different developmental trajectories related to caste, the higher respiratory rates observed in queens was due to greater concentrations of respiratory chain enzymes [81]. More recently, the over-expression

of metabolic enzyme transcripts by queen-destined larvae has been confirmed [17]. However, it has been suggested that these findings are purely a reflection of the enhanced growth rate of queens during late larval development [56]. It is, however, plausible, that even the slight physiological changes that occur in early adulthood in queenless workers would also result in higher metabolic respiration, and thus, would enhance their metabolic enzyme expression. This notion is supported by the finding that the increased expression of metabolic genes was not apparent in queen larvae until the 5th instar [17], suggesting that if reproductive state were to be, in part, regulated by metabolic enzymes, it would be so in the later stages. Thus, young adult workers, though not physiologically primed to become queens, can become oviparous and it is plausibly the same suite of genes that is turned on both queens and reproductive workers.

3.4.6 Reproductive Groundplan Hypothesis

One explanation for the emergence of social phenotypes is that regulatory pathways in the solitary ancestors of today's social and eusocial insect species have become co-opted over evolutionary time to produce the behavioural and physiological traits that define societies [82,83]. The RGPH predicts that the physiological and behavioural traits found in social species mirror what would have been found in their solitary ancestors. Further, it was a decoupling of the ancestral reproductive cycle (i.e., the reproductive and non-reproductive phases) into two parts that has allowed for caste development and a strong reproductive division of labour [83]. Recently, the insulin/insulin-like signaling (IIS) pathway has been implicated in the RGPH [10], as

it plays important roles in regulating insect life span, reproductive state, growth, and metabolism [84,85]. A group of insulin-producing cells (IPCs) that control growth and sugar metabolism have been found in *Drosophila* brains [86], and it has been determined that there is cross-talk between the IIS pathway and hormonal systems controlling growth. Specifically, the IIS can affect the regulation of adipokinetic hormone [87], which is involved in lipid metabolism, as well as juvenile hormone and ecdysone, both of which have been implicated in the regulation of caste development and reproduction in honey bees [51,52,88-90]. This study's identification of multiple physiometabolic genes as having specific expression according to reproductive behaviour supports the general notion of caste development from the reproductive groundplan of a solitary ancestor. However, the finding that the majority of genes up-regulated in sterile individuals appear to be novel to *Apis*, as they lack obvious homology with *Drosophila* speaks against one of the major points of this evolutionary theory. Rather than a simple decoupling of regulatory signals, and a reorganization of pre-established pathways, honey bee caste development appears to have required the establishment of novel genes over evolutionary time, with functions specific to social behaviour. In light of this finding, the data presented here both supports and refutes the RGPH, in that genes upregulated in reproductive workers appear to be expressed (and scientifically described) in *D. melanogaster*, which is both solitary and a more well-described and studied model. Conversely, very few identified genes showed homology with described genes in *D. melanogaster*. As the RGPH specifically discusses the evolutionary development of the caste system through to co-opting of previously lain pathways through changes in regulatory mechanisms, it predicts that

very few 'new' genes are needed [83,91,92]. As this study, as well as one other [9], has found that novel genes appear to correspond with both the (sterile) worker caste [9] and the sterile behavioural state within the worker caste (this study), it is thus predicted that gene expression patterns in the brains of honey bees while reproductively active are evidently ancestral, while groups genes expressed in those individuals who are sterile are novel to *Apis*, and may have evolved with eusociality.

3.5 Conclusion

In this experiment, worker reproductive behaviour was manipulated by a well-studied pheromonal cue [12,93]. Workers reared in the presence of their queen, or her pheromone, will maintain their ovaries in an inactive state. By contrast, workers reared in the absence of their queen, and thus insufficiently stimulated by her pheromones, will activate their ovaries and attempt to lay eggs. As these two reproductive states correspond with the reproductively 'selfish' and 'altruistic' states of the queen and worker castes, the model used in this study should detect differential gene expression correlated with reproductive altruism. On one hand, it seems that genes regulating metabolism are important for the initiation and maintenance of reproduction, and that the identification of well-described metabolic genes within the brains of 'queenless' reproductive workers would point to the unity between caste-related genes and genes involved more generally in insect development. The absence of homology between genes differentially expressed in the brains of 'queenright,' (or sterile) workers, which is the common state to find a worker in, and *Drosophila* suggests that the use of well studied, solitary insects to infer gene function in their

social species may be short-lived. However, with the sequencing of the honey bee genome [94], the availability of functional genomics resources [95], and the ability to manipulate honey bee behaviour and physiology [96] it is possible to establish gene function, model gene networks, and test evolutionary theories of behaviour.

References

1. Hölldobler B, Wilson EO (2009) The superorganism: the beauty, elegance, and strangeness of insect societies. W. W. Norton & Company Ltd. 522 p.

2. Hamilton WD (1964) The genetical evolution of social behaviour. I & II. Journal of Theoretical Biology 7: 1–52.

3. Queller DC, Strassmann JE (1998) Kin Selection and Social Insects. BioScience 48: 165–175.

4. Heimpel GE, de Boer JG (2007) Sex Determination in the Hymenoptera. Annual Review of Entomology 53: 209–230.

5. Crozier RH, Pamilo P (1996) Evolution of social insect colonies. Sex allocation and kin-selection. Oxford, UK: Oxford University Press. 306 p.

6. Crozier RH (2008) Advanced eusociality, kin selection and male haploidy. Australian Journal of Entomology 47: 2–8.

7. Seeley TD (2010) Honeybee Democracy. Princeton University Press. 280 p.

8. Winston ML (1991) The Biology of the Honey Bee. Harvard University Press. 281 p.

9. Barchuk AR, Cristino AS, Kucharski R, Costa LF, Simoes ZLP, et al. (2007) Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. BioMed Central Developmental Biology 7: 70.

10. de Azevedo SV, Hartfelder K (2008) The insulin signaling pathway in honey bee (*Apis mellifera*) caste development - differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. Journal of Insect Physiology 54: 1064–1071.

11. Grozinger CM, Fan Y, Hoover SER, Winston ML (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). Molecular Ecology 16: 4837–4848.

12. Hoover SER, Keeling C, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90: 477–480.

13. Amdam GV, Norberg K, Page REJ, Erber J, Scheiner R (2006) Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). Behavioral Brain Research 169: 201–205.

14. Page REJ, Amdam GV (2007) The making of a social insect: developmental architectures of social design. Bioessays 29: 334–343.

15. Hunt GJ, Amdam GV, Schlipalius D, Emore C, Sardesai N, et al. (2007) Behavioral genomics of honeybee foraging and nest defense. Naturwissenschaften 94: 247–267.

16. Amdam GV, Norberg K, Hagen A, Omholt S (2003) Social exploitation of vitellogenin. Proceedings of the National Academy of Sciences of the United States of America 100: 1799–1802.

17. Evans JD, Wheeler DE (2000) Expression profiles during honeybee caste determination. Genome Biology 2: research0001.1–0001.6.

18. Zhang B, Kirov S, Snoddy J (2005) WebGestalt: an integrated system for exploring gene sets in various biological contexts. Nucleic Acids Research 33: W741–W748.

19. Kanehisa M (2004) The KEGG resource for deciphering the genome. Nucleic Acids Research 32: 277D–D280.

20. Hoover SER, Higo H, Winston ML (2006) Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. Journal of Comparative Physiology Biology 176: 55–63.

21. Rosenzweig BA, Pine PS, Domon OE, Morris SM, Chen JJ, et al. (2004) Dye bias correction in dual-labeled cDNA microarray gene expression measurements. Environmental Health Perspectives 112: 1–8.

22. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467–470.

23. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, et al. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acid Research 30: e15.

24. Churchill G (2004) Using ANOVA to analyze microarray data. Biotechniques 37: 173–177.

25. Wu H, Kerr MK, Cui X, Churchill GA (2003) MAANOVA: A software package for the analysis of spotted cDNA microarray experiments. Statistics for Biology and Health 1: 313–341.

26. Kocher SD, Tarpy DR, Grozinger CM (2010) The effects of mating and instrumental insemination on queen honey bee flight behaviour and gene expression. Insect Molecular Biology 19: 153–162.

27. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 4: 44–57.

28. Alexa A, Rahnenfuhrer J, Lengauer T (2006) Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinformatics 22: 1600–1607.

29. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a pratical and powerful approach to multiple testing. Journal Of The Royal Statistical Society Series B-Methodological 57: 289–300.

30. Adams HA, Southey BR, Robinson GE, Rodriguez-Zas SL (2008) Meta-analysis of genome-wide expression patterns associated with behavioral maturation in honey bees. BioMed Central Genomics 9: 503.

31. Winston M, Slessor K (1992) Th essence of royalty - honey-bee queen pheromone. American Scientist 80: 374–385.

32. Thompson GJ, Kucharski R, Maleszka R, Oldroyd BP (2008) Genome-wide analysis of genes related to ovary activation in worker honey bees. Insect Molecular Biology 17: 657–665.

33. Thompson GJ, Kucharski R, Maleszka R, Oldroyd BP (2006) Towards a molecular definition of worker sterility: differential gene expression and reproductive plasticity in honey bees. Insect Molecular Biology 15: 637–644.

34. Wilson EO (1971) The Insect Societies. Cambridge, Massachusetts: Harvard University Press. 548 p.

35. Toth AL, Varala K, Henshaw MT, Rodriguez-Zas SL, Hudson ME, et al. (2010) Brain transcriptomic analysis in paper wasps identifies genes associated with behaviour across social insect lineages. Proceedings of the Royal Society B 277: 2139–2148.

36. Weil T, Korb J, Rehli M (2009) Comparison of queen-specific gene expression in related lower termite species. Molecular Biology and Evolution 26: 1841–1850.

37. Matsui T, Yamamoto T, Wyder S, Zdobnov EM, Kadowaki T (2009) Expression profiles of urbilaterian genes uniquely shared between honey bee and vertebrates. BioMed Central Genomics 10: 17.

38. Coghlan V, Perrino B, Howard M, Langeberg L, Hicks J, et al. (1995) Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science 267: 108–111.

39. Burg MG, Sarthy PV, Koliantz G, Pak WL (1993) Genetic and molecular

identification of a *Drosophila* histidine decarboxylase gene required in photoreceptor transmitter synthesis. European Molecular Biology Organisation 12: 911–919.

40. Blenau W, Baumann A (2001) Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaste*r and *Apis mellifera*. Archives of Insect Biochemistry and Physiology 48: 13–38.

41. Schwartz JC, Arrang JM, Garbarg M, Pollard H, Ruat M (1991) Histaminergic transmission in the mammalian brain. Physiological Reviews 71: 1–51.

42. Nassel DR (1996) Neuropeptides, amines and amino acids in an elementary insect ganglion: Functional and chemical anatomy of the unfused abdominal ganglion. Progress in Neurobiology 48: 325–420.

43. Boulay R, Hopper-Bui LM, Woodring J (2001) Ovipoisition and oogenesis in virgin fire ant females *Solenopsis invicta* are associated with a high level of dopamine in the brain. Physiological Entomology 26: 294–299.

44. Harris JW, Woodring J (1995) Elevated brain dopamine levels associated with ovary development in queenless worker honey bees (*Apis mellifera* L.). Comparative Biochemistry and Physiology 111: 271–279.

45. Baker DA, Russell S (2009) Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapause. BioMed Central Genomics 10: 242.

46. Kimura KD, Tisselbaum HA, Liu Y, Ruvkun G (1997) *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science 277: 942–946.

47. Flannagan RD, Tammariello SP, Joplin KH, Cikra-Ireland RA, Yocum GD, et al. (1998) Diapause-specific gene expression in pupae of the flesh fly *Sarcophaga crassipalpis*. Proceedings of the National Academy of Sciences of the United States of America 95: 5616–5620.

48. Tsai C-C, Kao H-Y, Yao T-P, Mckeown M, Evans RM (1999) SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. Molecular Cell 4: 175–186.

49. Li MA, Alls JD, Avancini RM, Koo K, Godt D (2003) The large Maf factor Traffic Jam controls gonad morphogenesis in *Drosophila*. Nature Cell Biology 5: 994–1000.

50. Amdam GV, Nilsen K-A, Norberg K, Fondrk MK, Hartfelder K (2007) Variation in endocrine signaling underlies variation in social life history. American Naturalist 170: 37–46.

51. Maekawa K, Ishitani K, Gotoh H, Cornette R, Miura T (2010) Juvenile hormone titre and vitellogenin gene expression related to ovarian development in primary reproductives compared with nymphs and nymphoid reproductives of the termite *Reticulitermes speratus*. Physiological Entomology 35: 52–58.

52. Hartfelder K (2000) Insect juvenile hormone: from "status quo" to high society. Brazilian Journal of Medical and Biological Research 33: 157–177.

53. Benyajati C, Place AR, Powers DA, Sofer W (1981) Determination in oocytes of the reproductive modes for the brine shrimp *Artemia parthenogenetica*. Proceedings of the National Academy of Sciences of the United States of America 78: 2717–2721.

54. Murtha MT, Cavener DR (1989) Ecdysteroid regulation of glucose dehydrogenase and alcohol dehydrogenase gene expression in *Drosophila melanogaster*. Developmental Biology 135: 66–73.

55. Guidugli KR, Hepperle C, Hartfelder K (2004) A member of the short-chain dehydrogenase/reductase (SDR) superfamily is a target of the ecdysone response in honey bee (*Apis mellifera*) caste development. Apidologie 35: 37–47.

56. Corona M, Estrada E, Zurita M (1999) Differential expression of mitochondrial genes between queens and workers during caste determination in the honeybee *Apis mellifera*. Journal of Experimental Biology 202: 929–938.

57. Thompson GJ, Paar JR, Kucharski R, Maleszka R, Oldroyd BP (n.d.) A genetic locus for worker sterility in a social insect. Unpublished. .

58. Montague CE, Oldroyd BP (1998) The evolution of worker sterility in honey bees: an investigation into a behavioral mutant. Evolution 52: 1408–1415.

59. Oldroyd BP, Osborne KE (1999) The evolution of worker sterility in honeybees: the genetic basis of failure of worker policing. Proceedings of the Royal Society B 266: 1335–1339.

60. Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R (2006) Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees. Genome Research 16: 1385–1394.

61. Maleszka R (2008) Epigenetic integration of environmental and genomic signals in honey bees. Epigenetics 3: 188–192.

62. Li J, Wu J, Begna Rundassa D, Song F, Zheng A, et al. (2010) Differential protein expression in honeybee (*Apis mellifera* L.) larvae: underlying caste differentiation. Public Library of Science ONE 5: e13455.

63. Li Y, Zhang Z, Robinson GE, Palli SR (2007) Identification and

characterization of a juvenile hormone response element and its binding proteins. Journal of Biological Chemistry 282: 37605–37617.

64. Pan ML, Wyatt GR (1971) Juvenile hormone induces vitellogenin synethsis in the monarch butterfly. Science 174: 503–505.

65. Cho W-L, Kapitskaya MZ, Raikhel AS (1995) Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. Insect Biochemical and Molecular Biology 25: 19–27.

66. Tobe SS, Pratt GE (1974) Influence of substrate concentrations on the rate of insect juvenile hormone biosynthesis by corpora allata of the desert locust *in vitro*. Journal of Biochemistry 144: 107–113.

67. Gade G, Hoffman K-H (2005) Neuropeptides regulating development and reproduction in insects. Physiological Entomology 30: 103–121.

68. Klowden MJ (2007) Physiological Systems in Insects. Second Ed. Boston: Academic Press. 688 p.

69. Lezzi M, Bergman T, Mouillet JF, Henrich VC (1999) The ecdysone receptor puzzle. Arch. Insect Biochem. Physiol. 41: 99–106.

70. Song Q, Gilbert LI (1995) Multiple phosphorylation of ribosomal protein S6 and specific protein synthesis are required for prothoracicotropic hormone-stimulated ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. Insect Molecular Biology 25: 591–602.

71. Kawasaki H, Kimihiko S, Quan G-X, Nohata J, Mita K (2003) Analysis of α- and β-tubulin genes of *Bombyx mori* using an EST database. Insect Biochemistry and Molecular Biology 33: 131–137.

72. Iwami M (2000) Bombyxin: an insect brain peptide that belongs to the insulin family. Zoological Science 17: 1035–1044.

73. Fullbright G, Lacy ER, Bullesbach EE (1997) The prothoracicotropic hormone bombyxin has specific receptors on insect ovarian cells. European Journal of Biochemistry 245: 774–780.

74. Yao T-P, Forman BM, Jiang Z, CHerbas L, Chen J-D, et al. (1993) Functional ecdysone receptor in the product of *EcR* and *Ultraspiracle* genes. Nature 366: 476–479.

75. Huang Z-Y, Robinson GE, Tobe SS, Yagi KJ, Strambi C, et al. (1991) Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. Journal of Insect Physiology 37: 733–741.

76. Schulz DJ, Sullivan JP, Robinson GE (2002) Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies. Hormones and Behavior 42: 222–231.

77. Hartfelder K, Bitondi MMG, Santana WC, Simões ZLP (2002) Ecdysteroid titer and reproduction in queens and workers of the honey bee and of a stingless bee: loss of ecdysteroid function at increasing levels of sociality? Insect Biochemistry and Molecular Biology 32: 211–216.

78. Fernald RD, White RB (1999) Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Frontiers in Neuroendocrinology 20: 224–240.

79. Herbison AE (1998) Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrine Reviews 19: 302–330.

80. Hunt JH, Nalepa CA (1994) Nourishment, evolution and insect sociality. In: Hunt JH, Nalepa CA, editors. Nourishment and Evolution in Insect Societies. Boulder, Colorado: Westview Press. pp. 1–19.

81. Eder J, Kremer JP, Rembold H (1983) Correlation of cytochrome C titre and respiration in *Apis mellifera*: adaptive response to caste determination defines workers, intercastes and queens. Comparative Biochemical Physiology 76: 703–716.

82. West-Eberhard M (1986) Alternative adaptations, speciation, and phylogeny (a review). Proceedings of the National Academy of Sciences of the United States of America 83: 1388–1392.

83. Amdam GV, Norberg K, Fondrk MK, Page REJ (2004) Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. Proceedings of the National Academy of Sciences of the United States of America 101: 11350–11355.

84. Wang Y, Amdam GV, Rueppell O, Wallrichs MA, Fondrk MK, et al. (2009) PDK1 and HR46 gene homologs tie social behavior to ovary signals. Public Library of Science ONE 4: e4899.

85. Drummond-Barbosa D, Spradling AC (2004) α-Endosulfine, a potential regulator of insulin secretion, is required for adult tissue growth control in *Drosophila*. Developmental Biology 266: 310–321.

86. Rulifson EJ, Kim SK, Nusse R (2002) Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science 296: 1118–1120.

87. Geminard C, Arquier N, Layalle S, Bourouis M, Slaidina M, et al. (2006) Control of metabolism and growth through insulin-like peptides in *Drosophila*. Diabetes 55: S5–S8.

88. Mackert A, do Nascimento AM, Bitondi MMG, Hartfelder K, Simoes ZLP (2008) Identification of a juvenile hormone esterase-like gene in the honey bee, *Apis mellifera* L. - Expression analysis and functional assays. Comparative Biochemistry and Physiology B 150: 33–44.

89. Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, et al. (2007) Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proceedings of the National Academy of Sciences of the United States of America 104: 7128–7133.

90. Soares MPM, Elias-Neto M, Simoes ZLP, Bitondi MMG (2007) A cuticle protein gene in the honeybee: Expression during development and in relation to the ecdysteroid titer. Insect Biochemistry and Molecular Biology 37: 1272–1282.

91. Amdam GV, Csondes A, Fondrk M, Page R (2006) Complex social behaviour derived from maternal reproductive traits. Nature 439: 76–78.

92. Amdam GV, Page RE, Fondrk MK, Brent CS (2010) Hormone response to bidirectional selection on social behavior. Evolution and Development 12: 428–436.

93. Mohammedi A, Paris A, Crauser D, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L.). Naturwissenschaften 85: 455–458.

94. Weinstock GM, Robinson GE, Gibbs RA, Worley KC, Evans JD, et al. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera.* Nature 443: 931–949.

95. Oldroyd BP, Thompson GJ (2007) Behavioural genetics of the honey bee *Apis mellifera.* Advances in Insect Physiology. 33: 1-49.

96. Robinson GE, Grozinger CM, Whitfield CW (2005) Sociogenomics: Social life in molecular terms. Nature Reviews Genetics 6: 257–270.

4. Discussion

The goal of my thesis was to review factors affecting worker reproductive altruism, and to identify genes associated with this behaviour in the highly social honey bee, *Apis mellifera*. I determined that both 'genetic' and 'environmental' factors contribute to reproductive status and behaviour of workers honey bees. However they did so in a hierarchical fashion, with environmental factors having a greater influence on the outcome of behaviour and physiology than underlying genetic factors, although both are clearly important. I was also able to identify new candidate genes for worker sterility by comparing gene expression in the brains of reproductively sterile and fecund workers, at four time-points during adult worker development. The functions of these genes were discussed with worker physiology and behaviour in mind.

Many field studies have attempted to control environmental background in order to better observe genetic difference effects among individual workers. For example, genetic strains or mutants reared within a common environment can be used to study the genetic basis of reproductive [1-3], guarding [4], and foraging behaviours [5-7]. In order to obtain populations of bees suitable for genetic studies, one or two genetic or environmental variables are manipulated, and gene expression differences are measured. As it stands, microarray and other expression data can be highly variable when studying natural populations with inherently wide variability in gene expression and behaviour [8]. This is particularly an issue within honey bee societies because of the complex signaling systems and the depth of redundancy within them. For example, both the reigning queen [3,9-13] and her brood [10,14-17] will prevent precarious egg-laying by normally sterile workers.

In this study, I have broken down published data into *genetic* and *environmental* components in an effort to describe the types of factors, and their interactions, which have influence on honey bee worker ovarian development.

In hindsight, the conclusion that environmental factors have a greater influence over worker ovarian development than the underlying genomic structure of an individual seems intuitive. Even in solitary insects there is an optimal time to put efforts into reproduction. For example, insects in temperate climates will not produce offspring until the temperature and day length have reached a certain threshold [18]. There may also be other cues involved, such as cycles in perennials, which may vary between regions [19]. It is also no surprise that breeding season will be synchronized with these important ecological factors [20]. As it is through the environmental interactions that natural selection occurs, and thus, genes will be selected for, or against, depending on what is optimal for an organism in their surrounding environment. And so, if reproductive cycle is directed by genes – which it is assumed to be – it would be beneficial for those controlling genes to be sensitive to the environmental indicators correlated with reproductive success. Therefore, although the genetic underpinnings of reproduction are necessary for oogenesis and successful oviposition, assuming all else equal, the ecological cues play a larger role in the reproductive behaviour of longer-lived insects, as was suggested by my meta-analytic review.

Rather than specifying behaviour directly, genes encode the proteins and peptides that govern the functioning of the brain . It is through these expressed molecular products that

behaviour distinct behaviours are produced. As discussed, it has been established that both brain development and organism behaviour depend on both genetic and environmental influences, including complex social cues [21]. For example, worker honey bees generally spend the first 2-to-3 weeks of their lives within the hive, caring for brood and maintaining the nest. Eventually they switch to collecting nectar and pollen. This occurs for the remainder of their lives (approx. 5 weeks). In the case of worker reproduction, their altruistic behaviour is a function of both gene expression and social context. It has been the goal of this study, and others, to identify candidate genes for the reproductive altruism displayed by these workers. As this study has demonstrated, metabolic genes are highly correlated with worker reproductive state, and have been found to interact with neurotransmitters [22] and hormones [23,24]. The insulin/insulin-like signaling (IIS) pathway has been implicated in one evolutionary theory for worker sterility and reproductive caste physiology, namely the Reproductive Groundplan Hypothesis (RGPH) [5,25]. The IIS pathway is also highly related to metabolism, specifically, glycolysis [26]. Other recent studies focusing on gene expression differences between queens and workers at the larval stages have also hinted at the importance of metabolism in honey bee reproductive physiology [2,27]. It is possible to take these genes lists, along with the lists produced by this study, to begin constructing the functional pathway that is responsive to social cues and ultimately regulates sterility and reproductive altruism. In order to understand sterility at the molecular level, the construction of this pathway is necessary.

Since, nutrition likely plays an important role in reproductive success and timing, the likelihood that metabolism genes are important for determining reproductive behaviour is high. However, the genes responsive to social cues within the hive (e.g., QMP and brood pheromone) that are responsible for the 'switch' in reproductive behavioural focus, from altruistic helper to selfish egg-layer, remain unknown. In order to establish physiological function within a social context, candidate genes identified by studies such as this one will have to be scrutinized more closely through knock-down (e.g., RNA interference) or protein-protein interaction (e.g., co-immunoprecipitation) experiments. ADH is likely a prime candidate in this regard due to its consistent expression pattern at various ages. However, with the sequencing of the honey bee genome [28], the availability of functional genomics resources [29], and the ability to manipulate honey bee behaviour and physiology [30] it is possible to establish gene function, model gene networks, and test evolutionary theories of social behaviour.

REFERENCES

1. Corona M, Estrada E, Zurita M (1999) Differential expression of mitochondrial genes between queens and workers during caste determination in the honeybee *Apis mellifera*. Journal of Experimental Biology 202: 929–938.

2. Evans JD, Wheeler DE (2000) Expression profiles during honeybee caste determination. Genome Biology 2: research0001.1–0001.6.

3. Grozinger CM, Fan Y, Hoover SER, Winston ML (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). Molecular Ecology 16: 4837–4848.

4. Guzman-Novoa E, Hunt GJ, Uribe-Rubio J, Prieto-Merlos D (2004) Genotypic effects of honey bee (*Apis mellifera*) defensive behavior at the individual and colony levels: the relationship of guarding, pursuing and stinging. Apidologie 35: 15–24.

5. Amdam GV, Omholt S (2003) The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. Journal of Theoretical Biology 223: 451–464.

6. Brockmann A, Annangudi SP, Richmond TA, Ament SA, Xie F, et al. (2008) Quantitative peptidomics reveal brain peptide signatures of behavior. Proceedings of the National Academy of Sciences of the United States of America 106: 2383–2388.

7. Amdam GV, Norberg K, Fondrk MK, Page REJ (2004) Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. Proceedings of the National Academy of Sciences of the United States of America 101: 11350–11355.

8. Whitfield CW, Cziko A, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302: 296–299.

9. Grozinger CM, Sharabash N, Whitfield CW, Robinson GE (2003) Pheromone-mediated gene expression in the honey bee brain. Proceedings of the National Academy of Sciences of the United States of America 100: 14519–14525.

10. Hoover SER, Winston ML, Oldroyd BP (2005) Retinue attraction and ovary activation: responses of wild type and anarchistic honey bees (*Apis mellifera*) to queen and brood pheromones. Behavioural Ecolology and Sociobiology 59: 278–284.

11. Hoover SER, Keeling C, Winston ML, Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90: 477–480.

12. Grozinger CM, Fischer P, Hampton JE (2007) Uncoupling primer and releaser responses to pheromone in honey bees. Naturwissenschaften 94: 375–379.

13. Maisonnasse A, Beslay D, Crauser D, Plettner E, Le Conte Y (2010) New insights into honey bee (*Apis mellifera*) pheromone communication. Is the queen mandibular pheromone alone in colony regulation? Frontiers in Zoology 7: 18.

14. Smedal B, Brynem M, Kreibich CD, Amdam GV (2009) Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*). Journal of Experimental Biology 212: 3795–3801.

15. Alaux C, Le Conte Y, Adams HA, Rodriguez-Zas SL, Grozinger CM, et al. (2009) Regulation of brain gene expression in honey bees by brood pheromone. Genes, Brain and Behaviour 8: 309–319.

16. Maisonnasse A, Lenoir J-C, Beslay D, Crauser D, Le Conte Y (2010) E-beta-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*). Public Library of Science ONE 5: e13531.

17. Jay SC (1972) Ovary development of worker honeybees when separated from worker brood by various methods. Canadian Journal of Zoology 50: 661–664.

18. Allen MJ (2007) What makes a fly enter diapause? Fly (Austin) 1: 307–310.

19. Thompson J (1991) Evolution of oviposition behavior and host preference in Lepidoptera. Annual Review of Entomology 36: 65–89.

20. Björkman C, Larsson S (1997) Oviposition preferences in pine sawflies: a trade-off between larval growth and defense against natural enemies. Oikos 79: 45–52.

21. Robinson GE, Fernald RD, Clayton DF (2008) Genes and social behavior. Science 322: 896–900.

22. Nassel DR (1996) Neuropeptides, amines and amino acids in an elementary insect ganglion: Functional and chemical anatomy of the unfused abdominal ganglion. Progress in Neurobiology 48: 325–420.

23. Amdam GV, Page RE, Fondrk MK, Brent CS (2010) Hormone response to bidirectional selection on social behavior. Evolution and Development 12: 428–436.

24. Hartfelder K (2000) Insect juvenile hormone: from "status quo" to high society. Brazilian Journal of Medical and Biological Research 33: 157–177.

25. West-Eberhard M (1986) Alternative adaptations, speciation, and phylogeny (a review). Proceedings of the National Academy of Sciences of the United States of America 83: 1388–1392.

26. Geminard C, Arquier N, Layalle S, Bourouis M, Slaidina M, et al. (2006) Control of metabolism and growth through insulin-like peptides in *Drosophila*. Diabetes 55: S5–S8.

27. Barchuk AR, Cristino AS, Kucharski R, Costa LF, Simoes ZLP, et al. (2007) Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. BioMmed Central Developmental Biology 7: 70.

28. Weinstock GM, Robinson GE, Gibbs RA, Worley KC, Evans JD, et al. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931–949.

29. Oldroyd BP, Thompson GJ (2007) Behavioural genetics of the honey bee *Apis mellifera*. Advances in Insect Physiology. 33: 1-49.

30. Robinson GE, Grozinger CM, Whitfield CW (2005) Sociogenomics: Social life in molecular terms. Nature Reviews Genetics 6: 257–270.

Appendix A: Chapter 3 Detailed Results

Table A.1 Genes that were differentially expressed in QMP+ and QMP- workers on consecutive days. The table shows the gene ID or accession number and the estimated fold-change. In addition, the table shows the inferred Biological Processes and Molecular Functions, using the fly ortholog (terms are separated with semi-colon, with Biological Process followed by Molecular Function). "Up" and "Down" refer to the regulation of genes according to the QMP-treatment group.

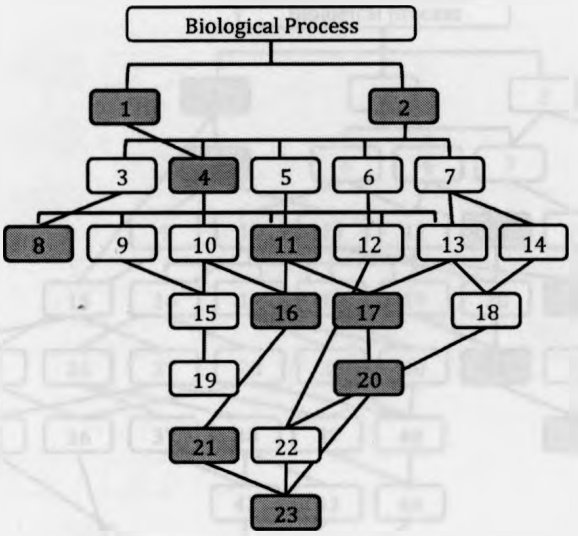
Gene Name	Fold-Change (Day 4)	Fold-Change (Day 6)	Fold-Change (Day 8)	Fold-Change (Day 10)	Biological Processes and Molecular Function	Fly Ortholog
Common elements in "Day 4" and "Day 6":						
Fbx13 (GB19733)	1.38 Up	1.43 Up			Unknown	FBgn0035959
Pyridoxine 5-phosphate oxidase (GB13619)	1.58 Up	1.72 Up			Vitamin metabolic process; Nucleotide binding	FBgn0030029
GB14629	1.18 Down	1.32 Down			Protein amino acid phosphorylation; Nucleotide binding	FBgn0025625
BI503065	1.52 Down	1.34 Down			Unknown	
Sepiapterin reductase (GB18184)	1.14 Up	1.50 Up			Unknown	
Myospheroid (GB12005)	1.17 Down	1.17 Down			Cell morphogenesis involved in differentiation; Cell adhesion molecule binding	FBgn0004657
GB13606	1.47 Down	2.26 Down			Unknown	
Anarchy 1 (GB14145)	1.17 Up	1.36 Up			Cell morphogenesis involved in differentiation; Unknown	FBgn0004055

Gene Name	Fold-Change (Day 4)	Fold-Change (Day 6)	Fold-Change (Day 8)	Fold-Change (Day 10)	Biological Processes and Molecular Function	Fly Ortholog
Germ cell-expressed bHLH-PAS	1.24 Down	1.44 Down			Regulation of transcription; DNA binding	FBgn0030627
GB14076	1.15 Down	1.06 Down			Unknown	FBgn0039727
BI506141	1.12 Down	1.16 Down			Unknown	
GB19480	1.23 Down	1.12 Down			Inositol metabolic process; nucleotide binding	FBgn0027279
Cactus (GB13520)	1.23 Down	1.26 Down			Immune system development, regulation of protein localization; transcription factor binding	FBgn0000250
Common elements in "Day 6" and "Day 8":						
Yellow-d (GB19132)		1.63 Up	1.34 Up		(MRJP-related)	FBgn0038151
GB13140		1.51 Up	1.31 Up		Arginine metabolic process; Ornithine-oxo-acid transaminase activity	FBgn0036381
Larval serum protein 2 (GB30362)		1.77 Up	1.11 Up		Unknown; Oxygen transporter activity	FBgn0002565
BI514253		1.07 Up	1.18 Up		Unknown	
BI516897		1.19 Up	1.18 Up		Unknown	
Protein lethal (2) essential for life (GB11383)		1.19 Up	1.12 Up		Response to temperature stimulus; identical protein binding	FBgn0011296
GB12679		1.24 Up	1.37 Up		Unknown	FBgn0034860
BI516696		1.30 Up	1.12 Up		Unknown	
Homeobox only domain (GB16549)		1.21 Up	1.19 Up		Eye development; Transcription factor activity	FBgn0005558
Queen brain-selective protein-1 (Qbp-1)		1.33 Up	1.21 Up		Unknown	

Gene Name	Fold-Change (Day 4)	Fold-Change (Day 6)	Fold-Change (Day 8)	Fold-Change (Day 10)	Biological Processes and Molecular Function	Fly Ortholog
Delta-aminolevulinic acid dehydratase (Porphobilinogen synthase) (GB17093)		1.25 Down	1.11 Down		Porphyrin metabolic process; Porphobilinogen synthase activity	FBgn0036271
Paxillin (GB19612)		1.25 Up	1.15 Up		Cytoskeleton organization; Zinc ion binding	FBgn0041789
GB19859		1.13 Down	1.05 Down		Unknown	
calcyphosine-like (GB17450)		1.23 Down	1.11 Down		Unknown	FBgn0011296
GB17905		1.13 Down	1.09 Down		Unknown	FBgn0034860
Common elements in "Day 8" and "Day 10":						
BI504328			1.07 Up	1.20 Up	Unknown	
GB14677			1.35 Up	1.09 Up	Purine metabolic process; glycine Hydroxymethyltransferase activity	FBgn0039241
Enhancer of rudimentary (GB17267)			1.22 Down	1.38 Down	Pyrimidine nucleotide metabolic process; Transcription regulator activity	FBgn0011586
Black (GB16827)			1.21 Down	1.05 Down	Pyrimidine base metabolic process; Aspartate 1-decarboxylase activity, Glutamate decarboxylase activity	FBgn0000153
GB20037			1.28 Up	1.26 Up	Unknown	FBgn0029838
GB30203			6.22 Up	2.38 Up	Intracellular protein transport; Unknown	FBgn0052654
Karst (GB15664)			1.36 Up	1.18 Up	Cytokinesis, Gamete generation; Actin binding, Microtubule binding	FBgn0004167
GB13199			1.19 Up	1.35 Up	Nucleoside metabolic process; Uridine phosphorylase activity	FBgn0039464
GB30316			1.27 Down	1.12 Down	Mesoderm development; Unknown	FBgn0039800

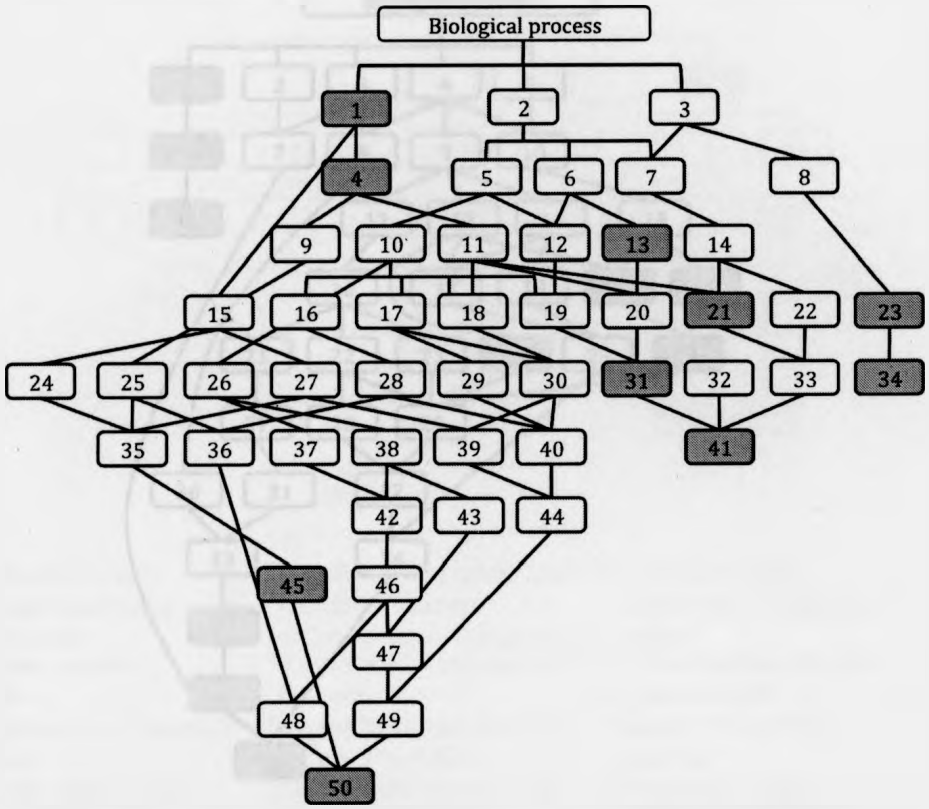
Gene Name	Fold-Change (Day 4)	Fold-Change (Day 6)	Fold-Change (Day 8)	Fold-Change (Day 10)	Biological Processes and Molecular Function	Fly Ortholog
Prolyl-4-hydroxylase-alpha EFB (GB15677)			1.20 Down	1.19 Down	Cellular amino acid derivative metabolic process; procollagen=proline 4-dioxygenase activity	FBgn0039776
GB19232			1.18 Up	1.25 Up	Mushroom body development, Oxidation reduction; Zinc ion binding	FBgn0031500
Monoacylglycerol O-acyltransferase 2 (GB13930)			1.22 Down	1.13 Down	Mesoderm development; Unknown	FBgn0033215
BI513124			1.18 Up	1.22 Up	Unknown	
DB761860			1.17 Up	1.14 Up	Unknown	
bolA-like 3 (GB16636)			1.28 Up	1.18 Up	Isoprenoid metabolic process; Purine nucleoside binding	FBgn0061360
Dauer Up-Regulated family member (dur-1) (BI512489)			1.19 Up	1.32 Up	Unknown	FBgn0015390
Death-associated protein 1 (DAP-1) (GB16354)			1.09 Up	1.14 Up	Unknown	FBgn0033624
GB16199			1.24 Up	1.20 Up	Unknown	
Common elements in "Day 4", "Day 6" and "Day 8":						
GB14940	1.26 Up	1.72 Up	1.40 Up		Arginine metabolic process; Ornithine-oxo-acid transaminase activity	FBgn0036381
GB19331	1.41 Up	1.43 Up	1.29 Up		Unknown	FBgn0021742
Chd64 (GB12375)	1.07 Up	1.47 Up	1.13 Up		Muscle system process; actin binding	FBgn0035499
Common elements in "Day 6", "Day 8" and "Day 10":						
GB10854		1.30 Up	1.16 Up	1.30 Up	Unknown; carboxylesterase activity	FBgn0015575
Smell impaired 21F (GB18993)		1.39 Up	1.20 Up	1.33 Up	Chemosensory behaviour, olfactory behaviour; Unknown	FBgn0016926

Gene Name	Fold-Change (Day 4)	Fold-Change (Day 6)	Fold-Change (Day 8)	Fold-Change (Day 10)	Biological Processes and Molecular Function	Fly Ortholog
15-hydroxy-prostaglandin dehydrogenase (GB18737)		1.62 Up	1.74 Up	1.73 Up	Endocytosis; Alcohol dehydrogenase (NAD) activity	FBgn0011693
Common elements in "Day 4", "Day 6", "Day 8" and "Day 10":						
GB15506	1.42 Up	1.65 Up	1.61 Up	2.27 Up	Oxidation Reduction; Unknown	FBgn0030332



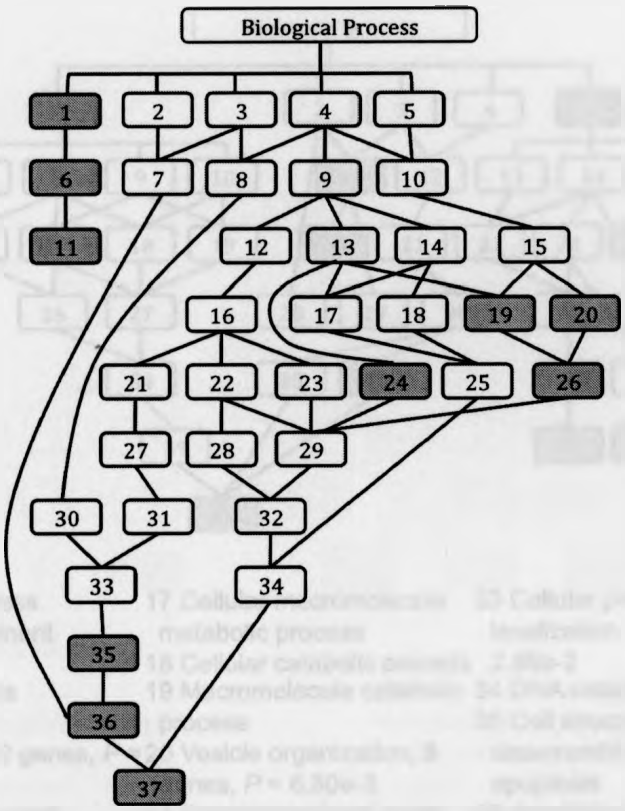
1 Cellular Process, 83 genes, P = 1.30e-3
2 Metabolic process, 69 genes, P = 1.56e-2
3 Macromolecule metabolic process
4 Cellular metabolic process, 60 genes, P = 1.30e-3
5 Catabolic process
6 Primary metabolic process
7 Nitrogen compound metabolic process
8 Cellular macromolecule metabolic process, 50 genes, P = 1.30e-3
9 Cellular ketone metabolic process
10 Organic acid metabolic process
11 Cellular catabolic process, 14 genes, P = 1.30e-3
12 Cellular amino acid and derivative metabolic process
13 Cellular nitrogen compound metabolic process
14 Amine metabolic process
15 Oxoacid metabolic process
16 Organic acid catabolic process, 5 genes, P = 4.80e-3
17 Cellular nitrogen compound catabolic process, 5 genes, P = 4.20e-3
18 Cellular amine metabolic process
19 Carboxylic acid metabolic process
20 Amine catabolic process, 5 genes, P = 2.50e-3
21 Carboxylic acid catabolic process, 5 genes, P = 4.80e-3
22 Cellular amino acid metabolic process
23 Cellular amino acid catabolic process, 5 genes, P = 1.90e-3

Figure A.1 Directed acyclic graphs of GO terms enriched in Day 4 QMP+ workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes (P < 0.05) were analysed for statistical enrichment of associated GO terms using fly orthologs.



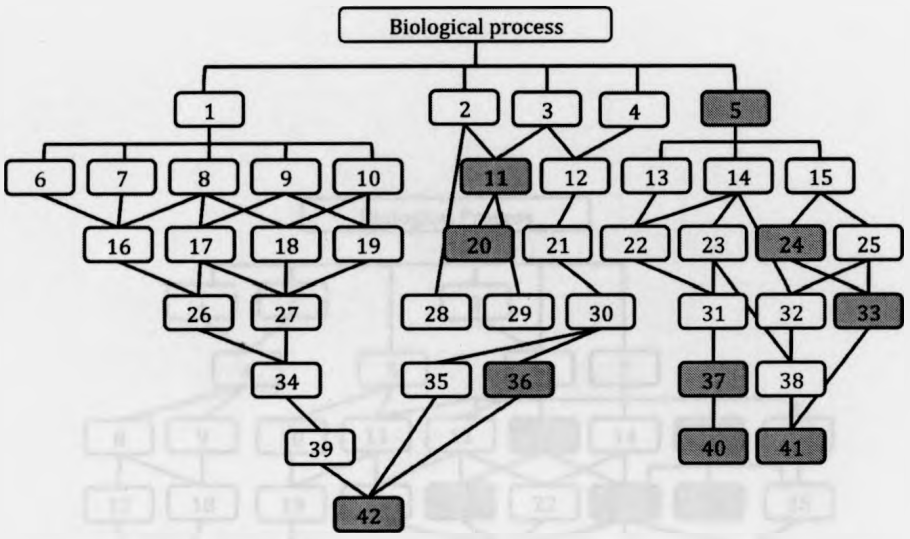
1 Biological regulation, 71 genes, P = 7.00e-4
2 Localization
3. Cellular process
4 Regulation of biological process, 68 genes, P = 7.00e-4
5 Establishment of localization
6 Cellular localization
7 Macromolecule localization
8 Cell communication
9 Positive regulation of biological process
10 Transport
11 Regulation of localization
12 Establishment of localization
13 Regulation of cellular process, 64 genes, P = 7.00e-4
14 Protein localization
15 Regulation of molecular function
16 Ion transport
17 Transmembrane transport
18 Regulation of transport
19 Intracellular transport
20 Regulation of cellular localization
21 Regulation of protein localization, 5 genes, P = 1.18e-2
22 Establishment of protein localization
23 Signal transduction, 33 genes, P = 1.18e-2
24 Positive regulation of molecular function
25 Positive regulation of transport
26 Cation transport
27 Regulation of transporter activity
28 Regulation of ion transport
29 Ion membrane transport
30 Regulation of transmembrane transport
31 Regulation of intracellular transport, 5 genes, P = 1.18e-2
32 Protein transport
33 Regulation of establishment of protein localization
34 Cell surface receptor linked signal transduction, 22 genes, P = 1.18e-2
35 Positive regulation of transporter activity
36 Positive regulation of ion transport
37 Di-, tri-valent inorganic cation transport
38 Metal ion transport
39 Regulation of transmembrane transporter activity
40 Regulation of ion transmembrane transport
41 Regulation of protein transport, 5 genes, 1.18e-2
42 Divalent metal ion transport
43 Regulation of metal ion transport
44 Regulation of ion transmembrane transporter activity
45 Positive regulation of ion transmembrane transporter activity, 2 genes, P = 1.18e-2
46 Calcium ion transport
47 Regulation of calcium ion transport
48 Positive regulation of calcium ion transport
49 Regulation of calcium ion transport via store-operated calcium channel activity
50 Positive regulation of calcium ion transport via store-operated calcium channel activity, 2 genes, P = 1.18e-2

Figure A.2 Directed acyclic graphs of GO terms enriched in Day 4 QMP- workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes ($P < 0.05$) were analysed for statistical enrichment of associated GO terms using fly orthologs.



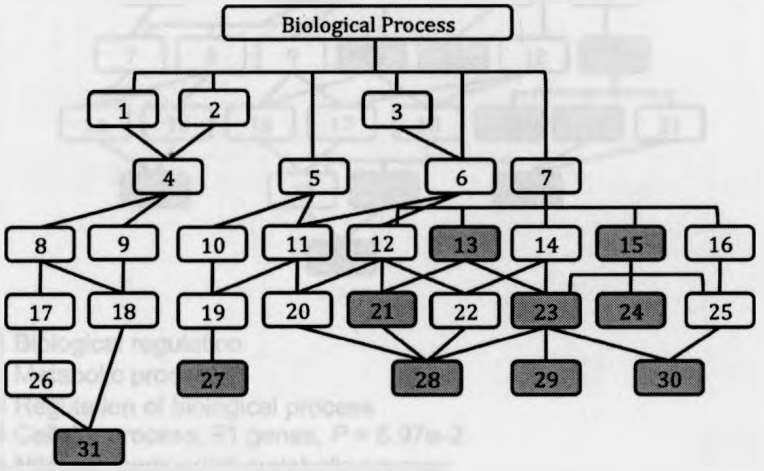
1 Biological regulation of genes, 84 genes, P = 7.40e-3
2 Cellular component organization
3 Cellular process
4 Developmental process
5 Multicellular organismal process
6 Regulation of biological process, 77 genes, P = 7.40e-3
7 Cell projection organization
8 Cellular developmental process
9 Anatomical structure development
10 Multicellular organismal development
11 Regulation of cellular process, 75 genes, P = 7.40e-3
12 System development
13 Anatomical structure morphogenesis
14 Appendage development
15 Post-embryonic development
16 Organ development
17 Appendage morphogenesis
18 Imaginal disc-derived appendage development
19 Post-embryonic morphogenesis, 21 genes, P = 7.40e-3
20 Instar larval or pupal development, 22 genes, P = 7.40e-3
21 Tissue development
22 Imaginal disc development
23 Organ morphogenesis
24 Metamorphosis, 21 genes, P = 7.40e-3
25 Imaginal disc-derived appendage morphogenesis
26 Instar larval or pupal morphogenesis, 21 genes, P = 7.40e-3
27 Ectoderm development
28 Wing disc development
29 Imaginal disc mprphogenesis
30 Cell differentiation
31 Epidermis development
32 Wing disc morphogenesis
33 Epidermal cell differentiation
34 Imaginal disc-derived wing morphogenesis
35 Hair cell differentiation, 6 genes, P = 7.40e-3
36 Non-sensory hair organization, 6 genes, P = 7.40e-3
37 Imaginal disc-derived wing hair organization, 6 genes, P = 7.40e-3

Figure A.3 Directed acyclic graphs of GO terms enriched in Day 6 QMP+ workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes ($P < 0.05$) were analysed for statistical enrichment of associated GO terms using fly orthologs.



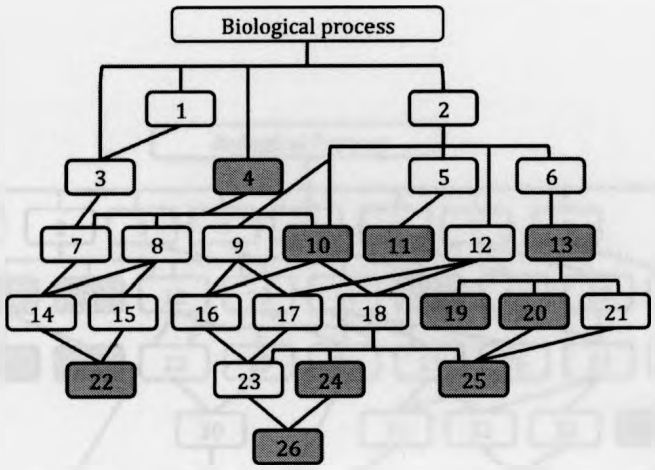
1 Metabolic process
2 Cellular component organization
3 Cellular process
4 Death
5 Localization, 42 genes, P = 3.44e-2
6 Nitrogen compound metabolic process
7 Primary metabolic process
8 Cellular metabolic process
9 Macromolecule metabolic process
10 Catabolic process
11 Organelle organization, 30 genes, P = 4.46e-2
12 Cell death
13 Cellular localization
14 Establishment of localization
15 Macromolecule localization
16 Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
17 Cellular macromolecule metabolic process
18 Cellular catabolic process
19 Macromolecule catabolic process
20 Vesicle organization, 6 genes, P = 6.80e-3
21 Programmed cell death
22 Establishment of localization in cell
23 Transport
24 Cellular macromolecule localization, 12 genes, P = 4.46e-2
25 Protein localization
26 DNA metabolic process
27 Cellular macromolecule catabolic process
28 Cellular component disassembly
29 Nucleus organization
30 Apoptosis
31 Intracellular transport
32 Establishment of protein localization
33 Cellular protein localization, 11 genes, P = 2.86e-2
34 DNA catabolic process
35 Cell structure disassembly during apoptosis
36 Apoptotic nuclear changes, 2 genes, P = 4.46e-2
37 Vacuolar transport, 4 genes, P = 1.91e-2
38 Protein transport
39 DNA catabolic process, endonucleolytic
40 Lysosomal transport, 3 genes, P = 5.16e-2
41 Intracellular protein transport, 11 genes, P = 1.91e-2
42 DNA fragmentation involved in apoptosis, 2 genes, P = 4.46e-2

Figure A.4 Directed acyclic graphs of GO terms enriched in Day 6 QMP- workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes ($P < 0.05$) were analysed for statistical enrichment of associated GO terms using fly orthologs.



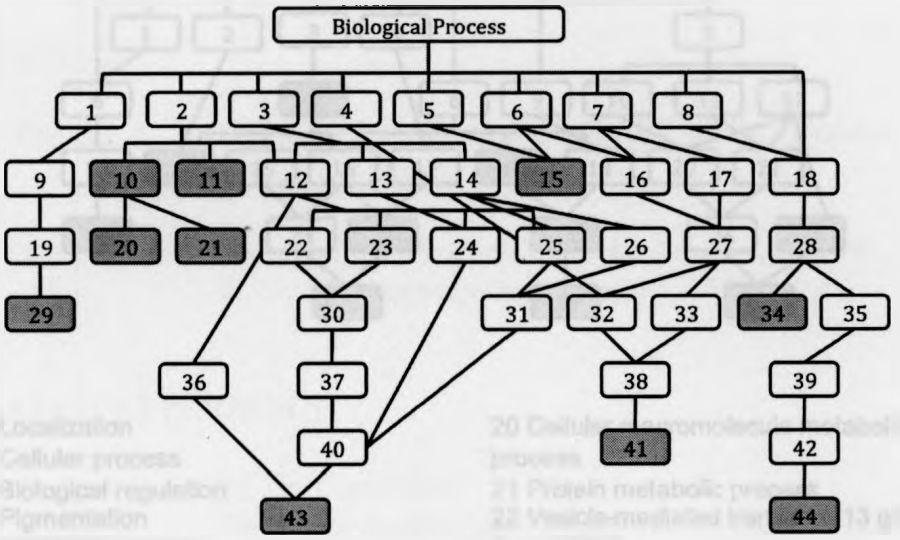
1 Multicellular organismal process
2 Developmental process
3 Biological regulation
4 Multicellular organismal development
5 Cellular process
6 Regulation of biological process
7 Metabolic process
8 Pattern specification process
9 Embryonic development
10 Cell communication
11 Regulation of cellular process
12 Regulation of metabolic process
13 Nitrogen compound metabolic process, 48 genes, P = 1.130e-3
14 Primary metabolic process
15 Cellular metabolic process, 67 genes, P = 5.59e-2
16 Macromolecule metabolic process
17 Regionalization
18 Embryonic pattern specification
19 Signal transduction
20 Regulation of cellular metabolic process
21 Regulation of nitrogen compound metabolic process, 22 genes, P = 7.89e-2
22 Regulation of primary metabolic process
23 Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, 41 genes, P = 4.10e-3
24 Heterocycle metabolic process, 10 genes, P = 7.89e-2
25 Cellular macromolecule metabolic process
26 Segmentation
27 Two-component transduction system (phosphorelay), 2 genes, P = 7.89e-2
28 Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, 22 genes, P = 7.89e-2
29 Nucleobase, nucleoside and nucleotide metabolic process 10 genes, P = 5.59e-2
30 RNA metabolic process, 26 genes, P = 7.89e-2
31 Segment specification, 5 genes, P = 4.66e-2

Figure A.5 Directed acyclic graphs of GO terms enriched in Day 8 QMP+ workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes (P < 0.05) were analysed for statistical enrichment of associated GO terms using fly orthologs.



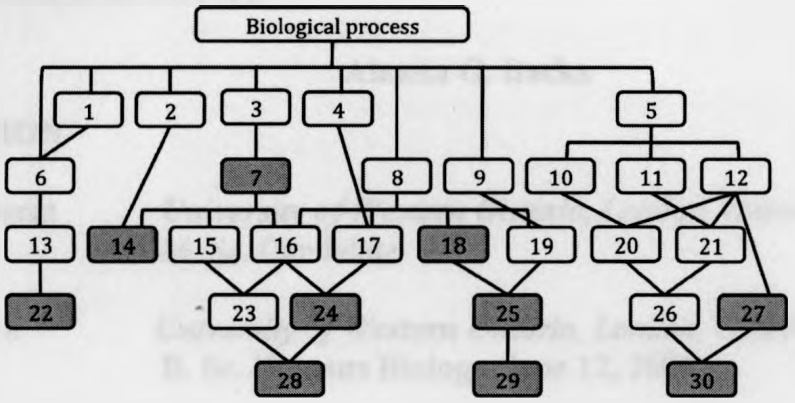
1 Biological regulation
2 Metabolic process
3 Regulation of biological process
4 Cellular process, 91 genes, P = 5.97e-2
5 Nitrogen compound metabolic process
6 Primary metabolic process
7 Regulation of cellular process
8 Cell cycle
9 Biosynthetic process
10 Cellular metabolic process, 60 genes, P = 8.13e-2
11 Amine metabolic process, 11 genes, P = 7.97e-2
12 Macromolecule process
13 Carbohydrate metabolic process, 14 genes, P = 5.97e-2
14 Regulation of cell cycle
15 Mitotic cell cycle
16 Cellular biosynthetic process
17 Macromolecule biosynthetic process
18 Cellular macromolecule metabolic process
19 Carbohydrate biosynthetic process, 5 genes, P = 5.97e-2
20 Cellular carbohydrate metabolic process, 9 genes, P = 5.97e-2
21 Polysaccharide metabolic process
22 Regulation of mitotic cell cycle, 6 genes, P = 7.97e-2
23 Cellular macromolecule biosynthetic process
24 Glycoprotein metabolic process, 5 genes, P = 7.97e-2
25 Cellular polysaccharide metabolic process, 3 genes, P = 7.97e-2
26 Glycoprotein biosynthetic process, 5 genes, P = 7.97e-2

Figure A.6 Directed acyclic graphs of GO terms enriched in Day 8 QMP- workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes (P < 0.05) were analysed for statistical enrichment of associated GO terms using fly orthologs.



1 Biological regulation
2 Cellular process
3 Metabolic process
4 Immune system process
5 Growth
6 Multicellular organismal process
7 Developmental process
8 Localization
9 Regulation of biological quality
10 Cell cycle, 20 genes, P = 2.97e-1
11 Cell proliferation, 8 genes, P = 2.97e-1
12 Cellular metabolic process
13 Primary metabolic process
14 Nitrogen compound metabolic process
15 Multicellular organism growth, 3 genes, P = 2.97e-1
16 Multicellular organismal development
17 Anatomical structure development
18 Establishment of localization
19 Homeostatic process
20 Cell cycle process, 17 genes, P = 2.97e-1
21 Mitotic cell cycle, 14 genes, P = 2.97e-1
22 Cellular ketone metabolic process
23 Organic acid metabolic process
24 Cellular amino acid and derivative metabolic process
25 Cellular nitrogen compound metabolic process
26 Amine metabolic process
27 System development
28 Transport
29 Anatomical structure homeostasis, 3 genes, P = 2.97e-1
30 Oxoacid metabolic process
31 Cellular amine metabolic process
32 Immune system development
33 Organ development
34 Organic acid transport, 4 genes, P = 2.97e-1
35 Ion transport
36 Cellular aromatic compound metabolic process
37 Carboxylic acid metabolic process
38 Hemopoietic or lymphoid organ development
39 Anion transport
40 Cellular amino acid metabolic process
41 Hemopoiesis, 4 genes, P = 2.97e-1
42 Inorganic anion transport
43 Aromatic amino acid family metabolic process, 3 genes, P = 2.97e-1
44 Phosphate transport, 2 genes, P = 2.97e-1

Figure A.7 Directed acyclic graphs of GO terms enriched in Day 10 QMP+ workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes ($P < 0.05$) were analysed for statistical enrichment of associated GO terms using fly orthologs.



1 Localization
2 Cellular process
3 Biological regulation
4 Pigmentation
5 Metabolic process
6 Establishment of localization
7 Regulation of biological process, 42 genes, *P* = 1.61e-2
8 Developmental process
9 Multicellular organismal process
10 Cellular metabolic process
11 Primary metabolic process
12 Macromolecule metabolic process
13 Transport
14 Regulation of cellular process, 33 genes, *P* = 1.98e-2
15 Negative regulation of biological process
16 Regulation of developmental process
17 Pigmentation during development
18 Aging, 7 genes, *P* = 9.30e-3
19 Multicellular organismal development
20 Cellular macromolecule metabolic process
21 Protein metabolic process
22 Vesicle-mediated transport, 13 genes, *P* = 1.23e-2
23 Negative regulation of developmental process
24 Regulation of pigmentation during development, 3 genes, *P* = 1.23e-2
25 Multicellular organismal aging, 7 genes, *P* = 9.30e-3
26 Cellular protein metabolic process
27 Macromolecule modification, 19 genes, *P* = 1.07e-2
28 Negative regulation of pigmentation during development, 2 genes, *P* = 1.91e-2
29 Determination of adult lifespan, 7 genes, *P* = 9.30e-3
30 Protein modification process, 18 genes, *P* = 1.23e-2

Figure A.8 Directed acyclic graphs of GO terms enriched in Day 10 QMP- workers. The top 10 significantly most enriched Biological Process terms (grey shading) are displayed in a hierarchical fashion, with parent terms at the top of the graph, and the lower, more specific, terms at the bottom of the graph. The list of upregulated genes ($P < 0.05$) were analysed for statistical enrichment of associated GO terms using fly orthologs.