



The Physiological and Genomic Bases of Bumble Bee Social Behaviour

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Abstract

Bumble bees are an outstanding model system in which to study the organization and evolution of complex social behaviour. Bumble bees pass through several distinct phases during their annual life cycle, including solitary and eusocial phases, and the final stage of the colony cycle is marked by intense competition among the queen and workers over the production of males. Furthermore, there are approximately 250 species of bumble bees, and of the few species that have been examined, it is clear that multiple life history strategies are possible. Thus, the ultimate and proximate mechanisms underlying co-operation, conflict, and behavioural plasticity can readily be examined in bumble bees. Here, we describe the current state of knowledge about the evolutionary, ecological, behavioural, physiological, chemical, and genomic mechanisms and factors underpinning bumble bee social behaviour throughout the colony cycle. We highlight long-standing questions in the field and discuss how advances in genomics and comparative approaches across bumble bee species can provide profound insights into this fascinating system and the evolution of social behaviour.

ABBREVIATIONS

CA	corpora allata
CP	competition phase
JH	juvenile hormone
Kr-h1	<i>Krüppel-homolog 1</i>
QLC	queenless compartment
Pre-CP	pre-competition phase
QEC	queen-excluded compartment
QL	queenless
QR	queenright
QRC	queenright compartment
Vg/vg	vitellogenin (protein/mRNA)



1. INTRODUCTION

Bumble bees are one of the most conspicuous bees in meadows of the temperate zones. They comprise about 250 species, all of which belong to

the genus *Bombus*, except for the social parasitic species that belong to the genus *Psithyrus*. Most bumble bees live in the temperate zones of the Northern hemisphere, but some species also occupy temperate zones of the Southern hemisphere and some are even present in tropical zones (Cameron et al., 2007; Michener, 1974; Sakagami, 1976; Williams et al., 2008). All species in the genus *Bombus* are social but are misleadingly considered ‘primitively’ eusocial due to their solitary mode of colony foundation, their annual colony life cycle, and because the queens possess pollen-collecting morphological characters. However, this classification is not completely accurate because in most species, castes are clearly recognizable by size and thus are morphologically distinct, and females also store food in cells different from the brood cells, both characteristics of highly eusocial bees (Goulson, 2010; Michener, 1974; Wilson, 1971).

Bumble bees are both economically important pollinators and have served as a principal model system for a variety of studies in social behaviour and ecology (Goulson, 2010). Moreover, with the development of new genomic tools and resources (Sadd et al., 2015), bumble bees are emerging as an outstanding model system to study the sociogenomic mechanisms mediating social behaviours. With few exceptions, bumble bee colonies have an annual life cycle, which encompasses first a solitary phase in which the queen cares for the first-generation brood, then a co-operative eusocial phase with a clear reproductive division of labour, and finally transitions to a period of intense competition and conflict. Thus, bumble bees can be used to study the proximate mechanisms that regulate complex social behaviour, as well as the ultimate mechanisms underlying the evolution of these behaviours.

Though there are hundreds of bumble bee species, studies have focused on only a handful of species, particularly *Bombus terrestris*. In this chapter, we will focus on the physiology and genomic mechanisms underlying social behaviour in *B. terrestris* and refer to other species whenever information is available.

1.1 The *B. terrestris* colony life history

The general descriptions of bumble bee life histories, nesting, and social behaviours were published before (Alford, 1978; Duchateau and Velthuis, 1988; Goulson, 2003; Michener, 1974). Here, we briefly outline their life cycle with emphasis on traits that might have an impact on their social behaviour and social physiology (Fig. 1). Since most of the studies pertaining to the physiology and genomics of bumble bees were performed with *B. terrestris*, we will first describe its life cycle, and thereafter allude to deviations from this general scheme with references to how it may affect social

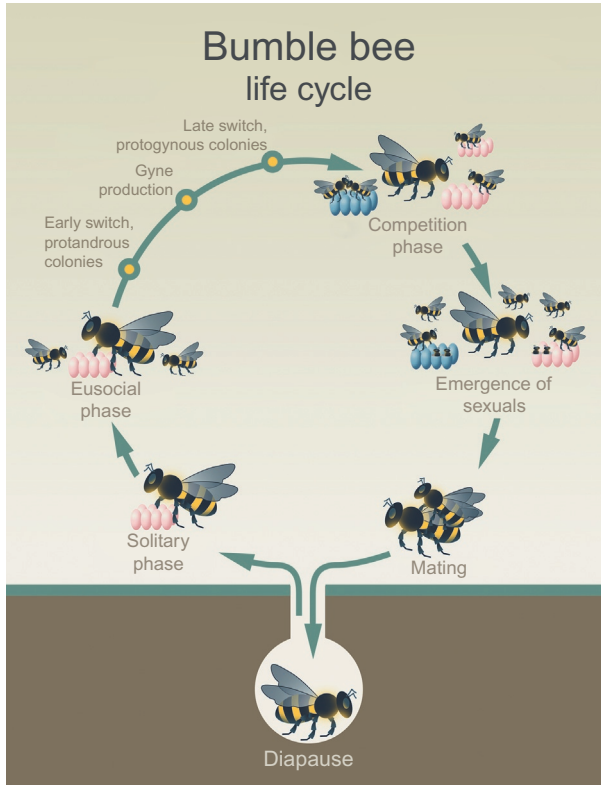


Figure 1 *Bombus terrestris* life cycle. Colonies rear one generation per year. New queens emerge near the end of colony cycle and leave the colony shortly after. During the solitary phase, the queens mate and enter a winter diapause that can last 6–9 months. Upon completion of diapause, queens forage, activate their ovaries, and lay their first brood (solitary phase). The colony's social phase begins with the emergence of the first worker (eusocial phase). The queen is the sole reproducer until the onset of the competition phase where females aggressively compete over male production. Males are produced at the 'switch point', where the queen switches from diploid egg to haploid egg production. The timing of the switch point varies between colonies and is likely influenced by ecological conditions. Gynes are produced towards the end of life cycle. Graphical design by Nick Sloff, Department of Entomology, Pennsylvania State University.

structure. It is also noteworthy that the geographical distribution of *B. terrestris* is very large, from Northern Europe to the Middle East, and probably encompasses several cryptic species or at least sub-species (Lecocq et al., 2013). This can explain the occasional differences in traits investigated by different laboratories that may have used different populations (e.g. Central Europe and Israel).

The colony cycle in *B. terrestris* starts with the diapause of mated queens, which can be initiated in the fall and lasts for 6–9 months (Alford, 1969b). Mated queens search for a subterranean cavity where they overwinter relying on their food reserves for survival (see Section 3.2, for more information). While in nature diapause is required to survive the winter, laboratory-reared queens can be induced to bypass diapause and are still able to produce colonies. These colonies, however, produce a greater number of gynes (future queens) (Beekman and van Stratum, 2000; Gosterit and Gurel, 2009). In nature, queens that skip diapause are bivoltine and are therefore selected to produce reproductives earlier in order for the next-generation queens to survive the approaching winter (see Section 1.3), and thus these laboratory-reared colonies may be reflecting a bivoltine life cycle.

After emerging from diapause, the founder queen searches for a nesting site (in *B. terrestris* nests are underground, usually in abandoned rodent burrows). Here, she builds several wax cells, provisions them with pollen, and lays the first batch of eggs (8–16 eggs). As the first batch of larvae develops, the queen continues foraging to provide them with pollen and nectar. When the larvae pupate, the queen typically builds additional egg cells on top of them to rear the second cohort of workers, which she continuously provides with pollen and nectar. This constitutes the *solitary phase* of the colony cycle.

In the next, *eusocial phase* of the colony, the queen stops foraging and is engaged solely in egg laying, while the workers take over all nest duties. During this phase, the nest population grows exponentially. After several generations of worker production and towards the end of the foraging season, reproductives (males and female gynes) are produced. Gyne production follows the ‘Bang–Bang’ strategy (Macevicz and Oster, 1976; Oster and Wilson, 1978), e.g., an abrupt shift from producing workers to producing gynes, consistent with *B. terrestris* having annual colonies and being semelparous (i.e. reproducing only once in a lifetime). Timing of male production in *B. terrestris* is more complex: depending on the colony, male production either precedes that of gyne production (protandry), or gyne production precedes that of male production (protogyny). There are also incidences of split sex ratios, i.e., within a population some colonies predominately produce gynes, whereas other colonies predominately produce males (Beekman and van Stratum, 1998; Bourke, 1997; Duchateau and Velthuis, 1988; Muller et al., 1992). The ecological and sociobiological factors underpinning these strategies will be discussed below. Males generally do not stay in the nest but disperse a few days post-emergence to establish mating territories, while gynes reside for a period of time in their maternal

nest before dispersing, mating, and seeking an appropriate location to diapause (Goulson, 2003).

1.2 The social history of the *B. terrestris* colony

Within the colony's *eusocial phase*, there are several distinct periods and events that have been revealed by behavioural observation of laboratory-reared colonies of *B. terrestris* (Duchateau and Velthuis, 1988). The first is the *harmonious period* during which the queen is the sole egg-layer and the workers assume all other nest duties. Behaviourally, this period is characterized by no observable conflict between the queens and workers, and an exponential ergonomic colony growth through the production of workers. It can also be considered the *pre-competition phase* (pre-CP, see below). The next event occurs when the queen switches her oviposition strategy, from laying diploid, female-destined, to haploid, male-destined eggs. Accordingly, the initiation of this phase was termed the *switch point*. Colonies that show protandry are described as 'early switch' colonies, and those exhibiting protogyny as 'late switch' colonies (Duchateau and Velthuis, 1988). Towards the end of the colony life cycle, nest harmony is disrupted because workers that possess activated ovaries concomitantly lay eggs and become aggressive not only towards the queen but also towards other reproductive workers. This period is termed the *competition phase* (CP). The CP can be readily recognized by the presence of several open egg cells. Typically, egg cells are sealed immediately after oviposition, but during the CP, competing workers or the queen may open each other's egg cells to cannibalize the eggs. The CP is also highly correlated with the switch in the developmental fate of the diploid larvae from worker-destined to queen-destined development (caste determination) (Cnaani et al., 2000a; Duchateau and Velthuis, 1988). The CP and emergence of reproductives mark the end of colony cycle, and after males and gynes disperse, the colony deteriorates rapidly.

1.3 Alternative life history strategies

The above described life and social histories are probably characteristic of most temperate bumble bee species, but deviations from the strict annual life cycle have been reported for several bumble bee species (Buttermore, 1997; Plowright and Lavert, 1984 and references therein; Sakagami, 1976; Skyrms et al., 2012; Stelzer et al., 2010). Indeed, even *B. terrestris* may be occasionally bivoltine under lab conditions (Beekman et al., 1999). How these alternative life history strategies impact the various social phases in the colony cycle

remains to be determined. The social structure of colonies of the tropical species, *B. atratus*, for example, is quite distinct (Cameron and Jost, 1998; Garofalo et al., 1986; cited in Sakagami, 1976; Zucchi, 1973). Colonies of this species seem to be perennial and present year round. One colony survived in the laboratory for 10 years, producing reproductives two to three times a year. While some of the mated young queens dispersed, other returned to the nest (in which the old queen died) creating a temporary polygynous nest. Polygyny however did not last long as the queens fought until a single reproductive queen remained. There is no mention of either worker aggression or worker reproduction. Unfortunately, these two detailed studies with *B. atratus* comprised only a single colony each, preventing any generalization.

1.4 Ultimate mechanisms shaping bumble bee social behaviour

Kin selection and inclusive fitness theory are considered the primary selection forces that shaped sociality, particularly in eusocial insect societies (Hamilton, 1964, 1972). However, this was recently challenged (Allen et al., 2013; Nowak et al., 2010; Wilson and Nowak, 2014), with the argument that relatedness among members of a eusocial colony is the consequence rather than the cause for sociality. Subsequent analyses demonstrated various flaws in this argument and re-validated the robust theoretical and empirical data supporting Hamilton's inclusive fitness theory for the evolution of eusociality (Abbot et al., 2011; Bourke, 2014). Inclusive fitness theory takes into account not only the degree of relatedness between the co-operating individuals but also group (or colony) productivity that results from such co-operation. Thus, for social insect colonies, reproductive output in terms of male and gyne production is a major factor (colony level selection), in addition to genetic relatedness, which affects the selection of social traits (Hammond and Keller, 2004). Thus, though relatedness may favour traits such as worker reproduction or queen nepotism, these traits cannot prevail if they hamper colony reproductive output because such colonies have lower fitness (reproductive output) than other conspecific colonies that do not express this trait. The evolution of social traits in social insects therefore reflects a delicate balance between individual and colony level selection. Below, we will discuss the fundamental role inclusive fitness and the resulting kin selection theory plays in the evolution of bumble bee social behaviour.

Most bumble bee species that have been studied are monogynous (a single queen per colony), the queen of which is monandrous (singly inseminated)

(Estoup et al., 1995; Owen and Whidden, 2013; Schmid-Hempel and Schmid-Hempel, 2000). As in all Hymenoptera, bumble bees have a haplodiploid sex determination system, where males are haploid and females are diploid. The queen is thus equally related to her male and female progeny ($r=0.5$) but is less related to worker-born males ($r=0.25$). She is therefore selected to monopolize the production of all progeny, males and females, as well as develop mechanisms that suppress worker reproduction. Workers, on the other hand, have more complex genetic gain. They are more related to their sister gynes ($r=0.75$) than to their would-be daughter gynes ($r=0.5$). It is therefore advantageous for the workers to rear sister gynes, which aligns with the queen's interest, resulting in harmony for gyne (and worker) production. In the case of male production, however, a worker is more related to her sons ($r=0.5$) than to her nephews (another nestmate worker's son $r=0.375$) or brothers (queen-born sons $r=0.25$) (Hamilton, 1972; Trivers and Hare, 1976). Therefore, kin selection predicts that workers are selected to compete with the queen and their sisters over male parentage. However, there are two limitations for worker reproduction: (1) workers must wait for the production of sufficient worker force before attempting to reproduce (according to the efficiency theory; Hammond and Keller, 2004) and (2) since workers gain more by rearing sister gynes than their own sons, they should be selected to defer reproduction until at least several diploid larvae became committed to gyne development (Alaux et al., 2005; Bourke and Ratnieks, 2001; Duchateau et al., 2004). Accordingly, queens can dominate male production in one of two ways: either coercively inhibit worker reproduction (queen control hypothesis; Keller and Nonacs, 1993) or control larval developmental fate and defer gyne production as late as possible in the colony cycle (given resource availability), thereby reducing the time period during which worker reproduction is favoured.



2. KIN CONFLICT OVER SEX RATIOS IN BUMBLE BEES

Inclusive fitness theory also provides a framework for explaining variation in male versus gyne production among bumble bee colonies. According to Trivers and Hare (1976) model of the sex ratio for the social Hymenoptera, a single, outbred, singly mated queen that is the sole reproducer (as in most bumble bee species) should equally invest in males and females (1:1), while workers are predicted to prefer females over males in a ratio of 3:1, consistent with the ratio of their relatedness to the reproductives ($r=0.75:0.25$ sister gynes:brothers). While sex investment ratios should

be either even or female biased (and accordingly closer to the queen or worker optimum), it is in practice clearly male biased (Bourke, 1997; Duchateau et al., 2004; Owen et al., 1980; Yeninar et al., 2000), with the early switching, protandrous colonies producing most of the males (Duchateau and Velthuis, 1988), posing an evolutionary question of both the proximate and ultimate reasons of this phenomenon. It was suggested that male bias is the result of worker reproduction in orphaned colonies (Owen et al., 1980), but male bias is present in both orphaned and queenright (QR) colonies (Paxton et al., 2001) and most of the males are queen-born (Alaux et al., 2004b; Brown et al., 2003a; Owen and Plowright, 1982; Paxton et al., 2001). It was further suggested that sexual selection for protandry in bumble bees leads to male-biased sex investment ratios (Bourke, 1997), and since both protandrous and protogynous colonies may exist within a protandrous population, it may result in bimodal sex variation with colonies producing either mostly males or mostly females, also termed as 'split sex ratio' (Beekman and van Stratum, 1998; Duchateau et al., 2004). Producing a queen is 1.69–2.11 times as costly as producing a male (Duchateau et al., 2004). Thus, a split sex ratio is hypothesized to be the result of optimal allocation of resources, with colonies under low resource conditions showing a male-biased investment ratio, whereas colonies under high resource conditions allocate more resources towards females (Beekman and van Stratum, 1998).

Thus, ecological and social constraints interact to shape the annual life cycle of *B. terrestris*. Since this species cannot store resources for long-term survival, the colony is unable to survive the harsh winter and must have an annual life cycle. Thus, neither the queen nor the workers have interest in deferring the conflict to later years (Bulmer, 1981), which allows aggression to dominate the final phase of the colony cycle. Finally, resource availability can determine the timing of the switch point and CP within the colony and the resulting sex ratio. Thus, this malleable annual colony cycle provides the researcher with an excellent opportunity to study the proximate mechanisms pertaining to queen–worker conflict.



3. BEHAVIOURAL, PHYSIOLOGICAL, AND GENETIC TRAITS ASSOCIATED WITH THE SOLITARY PHASE

3.1 Pre-mating and mating behaviour

The bumble bee queen undergoes several behavioural and physiological changes in the period after emergence and before diapause. The newly

emerged adult queens do not participate in colony tasks (Roseler and Roseler, 1988). Instead, they spend their first days accumulating fats and carbohydrates that are crucial for their ability to survive the winter diapause (Alford, 1969a; Fliszkievicz and Wilkaniec, 2007). When they are 6–7 days old, the gynes leave their mother colony (Roseler and Van Honk, 1990) and are attracted to a territorial pheromone produced by the labial glands of conspecific males who patrol in relatively fixed flight routes (Ayasse and Jarau, 2014; Bergman and Bergström, 1997; Kullenberg et al., 1970). Copulation is triggered by sex pheromones produced by the gynes' mandibular glands (Krieger et al., 2006; van Honk et al., 1978). In most of the studied bumble bee species, queens mate once (Estoup et al., 1995; Paxton et al., 2001; Schmid-Hempel and Schmid-Hempel, 2000). Among eight European species that were examined (including *B. terrestris*), only *Bombus hypnorum* was reported to be facultatively polyandrous (Brown et al., 2003a; Schmid-Hempel and Schmid-Hempel, 2000). Among the 11 North American species studied, 5 were reported to mate with multiple males (*Bombus bimaculatus*, *Bombus impatiens*, *Bombus mixtus*, *Bombus citrinus*, and *Bombus ternarius*) (Payne et al., 2003). *Bombus ignitus*, the species most studied in East Asia, was found to be monandrous (Takahashi et al., 2008), and in the tropical species *Bombus wilmattae*, seven of nine queens were singly mated and two were doubly mated (Huth-Schwarz et al., 2011b). Multiple mating can be advantageous to males (increased reproductive success), to queens (increase fertility), and to the colonies they produce (increased genetic diversity of the workers can improve efficiency and decrease pathogen load) (Arnqvist and Nilsson, 2000). Multiple mating can also have negative effects, and indeed, multiply mated queens had lower performance (diapause survival, female longevity, and fitness) as compared to singly mated queens (Baer and Schmid-Hempel, 2004). Indeed, although some bumble bee species are multiply mated, the effective mating number in bumble bees is close to one (Strassmann, 2001), suggesting post-mating mechanisms by which the sperm of one male is favoured. An inefficient mating plug that the male transfers to the queen during copulation and a trade-off between mating duration and the likelihood of re-mating were suggested as proximate factors underlying multiple mating in bumble bees (Brown et al., 2002; Payne et al., 2003).

Mating causes extensive short- and long-term physiological as well as behavioural modifications in females (Chapman et al., 1995; Gillott, 2003; Kocher et al., 2008). However, the mechanisms underlying post-mating changes in bumble bee queens have not been explored in great detail.

Ovarian activation (demonstrated by the average of the terminal oocytes in the ovaries, Fig. 2) is not induced by mating events (unlike in honey bee queens; Kocher et al., 2008); rather, ovaries remain inactive until diapause terminates, several months after mating. Furthermore, when kept separated from their natal colonies under laboratory conditions, virgin queens can lay haploid eggs that develop into males (Amsalem, E., personal observations). Levels of juvenile hormone (JH), the apparent gonadotropin in *B. terrestris*, are low in virgin queens and remain low in mated and diapause queens, increasing only several days after the termination of diapause (Larrere et al., 1993). The same is true for ecdysteroids (the exact identity of the ecdysteroid has not yet been elucidated for bumble bees), which remain low before and after mating (Geva et al., 2005). Although most of the queens that survive diapause are mated (Alford, 1969b), mating is not obligatory for entering diapause, and unmated queens are able to survive diapause (Greeff and Schmid-Hempel, 2008). Indeed, mating may have negative effects, since mated queens have reduced longevity and increased melanization

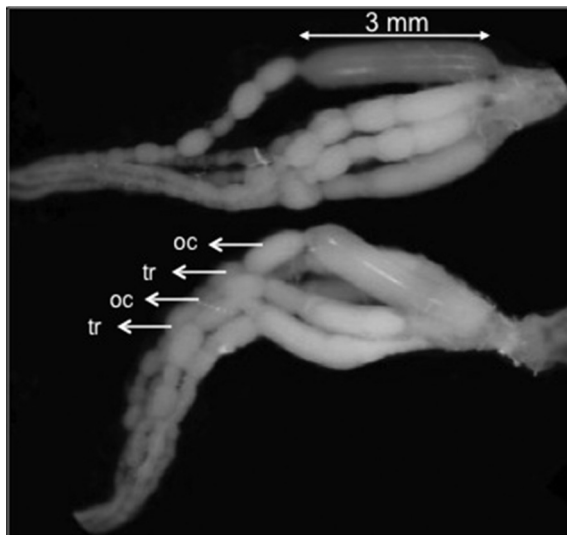


Figure 2 Ovaries of *Bombus terrestris* females. Workers and queens possess four ovarioles per ovary, for a total of eight ovarioles per individual. The length of the terminal oocyte is used to determine ovarian status. Either the average of the three largest oocytes (at least one ovariole per ovary) or the average of all of the eight terminal oocytes is equally effective in determining ovarian activation. Ready-to-lay eggs are approximately 3 mm long. Resorption of eggs appears as deformation in the shape of the terminal oocytes. Oc, oocyte (egg cell); tr, tropocyte (nursing cell).

of their spermatheca compared to virgin queens (Greeff and Schmid-Hempel, 2008), and multiply mated queens have reduced survival during diapause (Baer and Schmid-Hempel, 2004). Overall, mating was reported to have more negative than positive effects on queens, in contrast to the hypothesis that sexual conflict between males and females should be low in monandrous species (Strassmann, 2001).

3.2 Diapause in newly mated queens

Shortly after the queens mate, they enter a winter diapause that can last 6–9 months (Alford, 1969b). The factors regulating the onset and termination of diapause are still elusive. While diapause in most insects is generally triggered by temperature and/or day length (Denlinger, 2002; Denlinger et al., 2012; Sim and Denlinger, 2013), the onset of diapause in bumble bees is not likely to be induced by day length, while the role of temperature changes as prime cause for entering or exiting diapause remains to be examined (Alford, 1969b; Yeninar et al., 2000).

Environmental conditions, such as temperatures and diapause duration, interacting with queen nutritional stores, seem to be largely responsible for queen survival during diapause. *B. terrestris* queens exhibit lower survival rates at longer diapause (Beekman et al., 1998; Gosterit and Gurel, 2009; Yoon et al., 2010), whereas diapause temperature had either a negative effect on queen survival (Yoon et al., 2010) or no effect at all (Beekman et al., 1998). Although queens of *B. terrestris* below a threshold weight of 0.6 g did not survive diapause (different bumble bee species may have a different thresholds as function of the temperature of their habitats), above that threshold, no correlations were found between body mass and survival (Beekman et al., 1998) or between post-diapause queen weight and colony development (Gosterit and Gurel, 2007). Thus, queens need to reach a minimal threshold weight to survive diapause. This may also explain why workers cannot survive diapause: their small size does not allow them to store enough reserves to survive the long winter. Since the queen and worker castes only differ in body size, with queens being three times larger than the largest workers (Goulson, 2010; Michener, 1974), selection for body size, and thus the ability to survive diapause, was probably the first step in the divergence of castes in bumble bees and other primitively eusocial insects that go through winter diapause (Hunt et al., 2007). Indeed, size differences between the castes are simply the result of longer instar duration in prospective queen larvae (Cnaani and Hefetz, 2001). Thus, *B. terrestris*

workers are basically dwarf queens and size differences may be the result of a queen manipulation during larval development. Indeed, close contact with the queen was found to influence larval development and worker body size (Shpigler et al., 2013) and bees that were cared for by 10 workers without a queen were significantly larger than bees cared for by 10 workers with a queen (Shpigler et al., 2013).

Queens use a variety of nutritional reserves to survive diapause. The accumulation of sufficient quantity of fat body reserves prior to diapause, mainly lipids and glycogen, is critical (Fliszkiewicz and Wilkaniec, 2007). Beside glycogen and lipids, queens also store nectar in their crop prior to entering diapause. Up to 80% of the reserves are consumed during diapause (Alford, 1969b). It was suggested that the metabolism, although suppressed during diapause, still responds to temperature, which is consistent with the observed higher consumption of reserves during the first half of diapause, when temperatures are also higher. However, different temperatures during diapause in *B. terrestris* did not have a significant effect on queen survival (Beekman et al., 1998; Vesterlund et al., 2014; but see Yoon et al., 2010).

When mated queens are treated with CO₂, a method that was developed to bypass diapause and induce oogenesis (Roseler, 1985), their survival is higher and the time to initiate egg laying is shorter compared to non-narcotized non-diapause queens (Tasei, 1994). It was suggested that CO₂ narcosis inhibits the formation of fat body reserves and increases the size of the corpora allata (CA), the source of the gonadotropin JH (Roseler, 1985; Roseler and Roseler, 1984). A recent study corroborated that CO₂ narcosis decreases lipid mass and increases JH levels, as well as affects the expression levels of selected candidate genes related to reproduction, hormone response, immunity, stress, and neural response (Amsalem, E., Galbraith, D., Cnaani, J., Teal, P., Grozinger, C.M., unpublished data). However, the pertinent mechanisms underlying the effects of CO₂ narcosis remain to be elucidated.

Different diapause lengths or the lack of diapause (in either 'natural' non-diapausing (Beekman et al., 1999) or CO₂-treated queens) affects not only queen survival and physiology but may also affect long-term characteristics of colonies headed by such queens, including colony size and the number of males and gynes produced. However, several studies provide contradicting results pertaining to these effects. Lack of diapause affected queen characteristics; non-diapause non-narcotized queens produced the lowest number of workers but the highest number of gynes (Beekman and van Stratum, 2000) and non-diapause narcotized queens produced significantly more workers, males, and gynes than the diapause queens (Gosterit and Gurel, 2009). While

some studies did not find any differences between colonies headed by queens that were subjected to different diapause lengths (Beekman and van Stratum, 2000; Beekman et al., 1998; Gosterit and Gurel, 2009), another study found several differences: queens under longer diapause produced more brood during the first and the second generations, reached the switch point earlier, and produced colonies that reared more males and fewer gynes (Duchateau et al., 2004), consistent with the idea that colonies under low resource conditions are male biased (Beekman et al., 1998). However, in more recent studies, where the effect of diapause length on the number of reproductives produced was tested in controlled environments (under different colony size and photoperiod regimes), the effects seemed to be much more complicated: both colony size and diapause length affected the number of gynes (but not the number of males) and queen longevity in various ways and no interaction was found between colony size and diapause length (Amin et al., 2011). In a second study conducted by the same authors, gyne production was dependent on photoperiod but not on diapause duration, and both factors affected male production (Amin et al., 2007b). Different temperatures and photoperiodic regimes lead to different amount of weight loss in queens (Amin et al., 2007a; Yoon et al., 2010), and heavier queens produce larger brood in the first generation, but other colony parameters are not affected (Gosterit and Gurel, 2007). Several other factors such as pathogen and parasite loads may impact the queen post-diapause performances. For example, *Crithidia bombi*, a bumble bee parasite, had strong effect on survival and colony development: the pathogen was found to decrease the ability of queens to successfully establish a colony, colony size, number of males, and total fitness (Brown et al., 2003b). Infection with *Nosema bombi*, a gut parasite, did not affect colony structure but decreased the number of queens that successfully established a colony (Steen, 2008).

3.3 Genomic mechanisms underlying the queen solitary phase

Genomics studies of bumble bee queen behaviour and physiology have largely focused on the early stages of the queen's life cycle. A whole-body transcriptomics analysis demonstrated that several of the top 10 genes whose expression best differentiated virgin queens from males and workers are involved in nutrient storage (e.g. hexamerins) and fatty acid biosynthesis (Colgan et al., 2011); differential expression of these genes may prepare queens for diapause. Expressed sequence tag analysis of diapausing queen in *B. ignitus* revealed changes in expression levels of several candidate genes (Kim et al.,

2006). Another study focusing on three heat shock protein genes (*shsp*, *hsc70*, and *hsp90*) found dynamic changes in expression pattern that varied across the different queen tissues during diapause (Kim et al., 2008). We recently demonstrated that ~120 genes are consistently differentially expressed (either up- or down-regulated) in the fat bodies of diapausing queens relative to both mated and foundress queens, and these genes function in nutrient storage, metabolic processes, and stress resistance (Amsalem, E., Galbraith, D., Cnaani, J., Teal, P., Grozinger, C.M., unpublished data). Gene expression patterns in foundress queens differ substantially from virgin and mated queens in both the brain (Woodard et al., 2014) and fat body (Amsalem, E., Galbraith, D., Cnaani, J., Teal, P., Grozinger, C.M., unpublished data), respectively. However, when comparing laying queens in young colonies with and without workers, though the presence of workers substantially changed queen behaviour (queens laid more eggs and tended brood less), there was little effect of worker presence on queen brain gene expression (Woodard et al., 2013). Overall, these data indicate that there are substantial differences in gene expression profiles associated with the different physiological states that queens experience during their lives (virgin, mated, diapausing, egg laying), but other factors (perhaps changes in neuroendocrine signalling) underpin behavioural changes in laying queens in young colonies (e.g. the transition from both reproduction and maternal care to reproduction only). However, gene expression differences between pre-CP and CP queens have not been assessed, and this would be an exciting area for future research, particularly if queen-produced pheromones mediate caste differentiation in larvae. In honey bees, transcriptional profiles in pheromone-producing exocrine glands are strongly correlated with social environment (Malka et al., 2014).



4. BEHAVIOURAL, PHYSIOLOGICAL, AND GENETIC TRAITS ASSOCIATED WITH THE EUSOCIAL PHASE

As previously discussed, the *eusocial phase* in the bumble bee colony can be divided into two periods: the pre-CP and the CP. The pre-CP is characterized by social harmony and a complete reproductive division of labour between the queen and workers. In contrast, the CP is characterized by overt conflict over reproduction between the two castes. This provides an excellent opportunity to test evolutionary hypotheses stemming from kin selection as well as investigate the underlying proximate mechanisms.

There are two contrasting hypotheses regarding the ultimate cause for the sharp reproductive skew in social insects. The first was termed ‘queen

control' because it postulates that the queen coercively inhibits worker reproduction, either by behavioural or by pheromonal means. The second, termed 'queen signal' (or 'worker control'), postulates that workers have control over their own reproduction and behave, reproductively, in a manner that maximizes their own inclusive fitness, and the queen produces an honest signal of her reproductive abilities (Keller and Nonacs, 1993; Kocher and Grozinger, 2011). Since it is very difficult, if not impossible to discern between these two ultimate causes by proximate experiments, we will refrain from deciding which prevails but discuss the queen's effect on worker reproduction without referring to the ultimate cause.

4.1 Who wins the conflict over male production?

When discussing the outcome of the queen–worker conflict over male parentage, a distinction, at least for *B. terrestris*, between the pre-CP and the CP must be considered. All previous studies demonstrate that before the CP all eggs, irrespective of ploidy, are queen-produced. However, even in the pre-CP, a proportion of the workers possess activated ovaries (for *B. terrestris*, Alaux et al., 2004b; Amsalem et al., 2009; Duchateau and Velthuis, 1989; for *B. impatiens*, Cnaani et al., 2002; Jandt and Dornhaus, 2009) and thus apparently refrain from egg laying. Queen domination of male production also seems to prevail after the competition point. Behavioural observations followed by male genotyping showed that although over 38% of the workers were egg-layers, over 95% of the adult males were queen-born (Alaux et al., 2004b). This was also confirmed both by behavioural observation studies of laboratory-reared colonies (Duchateau and Velthuis, 1989) and by molecular studies of field-collected colonies (O'Connor et al., 2013). Two reasons for the queen domination of male production in *B. terrestris* were provided. First, the queen is far more fecund than workers and performed more active oophagy compared to workers (Alaux et al., 2004b; Bloch and Hefetz, 1999b). Second, during the CP, workers preferentially devour other worker's eggs, but not the queen's eggs (Zanette et al., 2012).

The outcome of queen–worker conflict over male parentage is more variable in the other species of bumble bees that have been examined. Queen domination of male production also seems to occur in *Bombus melnopygus* (Owen and Plowright, 1982) and *Bombus polaris* (Richards, 1977), but there was no information regarding the temporal dynamics of egg laying (e.g. before or during the CP). However, in the case of *B. hypnorum*, there is evidence that workers can successfully produce male

eggs, though this may vary with queen mating number. In the first study (Paxton et al., 2001), the queen was multiply mated (effective mating frequency of 1.26 harmonic mean) and male genotyping revealed that they were all queen-produced, while worker-laid eggs were presumably destroyed by the queen (under this effective mating frequency, worker policing is not selectively favourable; Ratnieks, 1988). In the second study (Brown et al., 2003a), all queens were singly inseminated, and over 20% of the males produced in QR colonies were worker derived. Thus, in colonies headed by singly mated queens, *B. hypnorum* workers are better competitors than *B. terrestris* workers. Finally, in the case of neotropical bumble bees, workers dominate male production in both *B. wilmatte* (Huth-Schwarz et al., 2011a) and *B. attratus* (Zucci, 1973). Thus, comparative studies with additional bumble bee species may provide great insight into the mechanisms mediating queen–worker conflict.

4.2 Underlying hormonal mechanisms and queen effect on worker reproduction

Before addressing the relative roles of the queen and workers in the above conflict and its resolution, it is useful to describe briefly the hormonal mechanisms underlying ovary activation and reproduction in *B. terrestris*. Several independent studies have demonstrated that in *B. terrestris*, similar to many non-social insect species, JH acts as a gonadotropin. In gynes, JH titers are low, which largely affects the queen's metabolism, directing it into lipid and carbohydrate storage rather than ovary activation. Experimentally elevating JH blocks the synthesis of these storage products and enhances ovary activation (Roseler and Roseler, 1988). Since JH titers are constantly high in functional, egg-laying queens (Bloch, 1997; Bloch et al., 2000a), it is more informative to study the role of JH and its dynamics in workers before and during ovary activation. Early studies have shown that when workers are rendered queenless (QL), JH levels rise (Roseler, 1977; Roseler and Roseler, 1978), but these studies used the *Galleria* bioassay, which is only semi-quantitative. A more quantitative assessment of JH levels was subsequently performed using the highly sensitive radioimmunoassay and examined both JH biosynthesis and secretion rates by the CA and its titers in the hemolymph (Bloch et al., 1996, 2000a). The gonadotropin role of JH was also recently confirmed in experiments where the CA were either surgically removed (Shpigler et al., 2014) or impaired by application of precocene-I (Amsalem et al., 2014c). All the above studies demonstrated that when workers are rendered QL, JH biosynthesis and JH hemolymph levels

increase which is followed by ovarian activation. Furthermore, activation of the CA and subsequent increase in JH titers induces the biosynthesis of the egg-yolk protein vitellogenin (Vg), which is then incorporated into the developing oocyte (Roseler and Roseler, 1988). However, the exact relationship between ovary activation, JH and Vg biosynthesis, and particularly the direct regulation of JH on Vg levels, are not fully resolved yet and will be discussed in Section 5.5.

Since in QR workers JH levels are low and ovaries inactive, it was postulated that the queen effect is manifested by inhibiting the activation of the CA and thereby blocking the subsequent cascade of events (reviewed in Roseler and Van Honk, 1990). To explain worker reproduction during the CP, it was postulated that the queen loses her inhibitory capacity, allowing the workers that have already developed ovaries, the so-called elite workers, to start laying eggs (Doorn van and Heringa, 1986). However, studies demonstrated that workers housed with mated pre-CP and CP queens had reduced JH titers and inactive ovaries relative to QL workers or workers housed with virgin queens (Bloch et al., 1996). Thus, in small groups, the inhibitory effect of the queen is intact. It is possible that as worker population increases near the end of the colony cycle, the queen's inhibitory capacity is diluted, which would be the case if the queen used aggression or pheromones to inhibit worker reproduction (see below, for further discussion). In a subsequent set of experiments, Bloch and Hefetz (1999b) measured worker reproduction, JH levels, and oocyte development in workers that were introduced into intact colonies at various stages of colony social development, before and after dequeening. In the pre-CP colonies before dequeening, both JH levels and oocyte activation in the introduced workers were low, indicative of reproductive inhibition. In contrast, after dequeening, workers introduced into pre-CP colonies had high JH and many of them had chorionated, ready-to-lay eggs. When introduced into colony at the verge of the CP, 90% of the introduced workers had activated ovaries, and a few had chorionated eggs even in the presence of the queen. Finally, nearly all workers introduced into colonies during the CP had low reproductive capacities, only about 4% had chorionated eggs. There was no difference in the distribution of ovary activation among the introduced workers whether the queen was present or absent. This indicates that during the CP, the workers that are already reproducing can inhibit reproduction of their sisters. The latter was further confirmed by the finding that old reproductive workers inhibit reproduction of introduced callow workers in small QL groups (Bloch and Hefetz, 1999b).

4.3 The chemistry underlying queen–worker conflict over reproduction—Is there a queen pheromone?

The queen mandibular gland secretion was the first candidate tested for its possible mediation of the queen inhibitory effect. Colonies headed by queens with extirpated mandibular glands started egg laying much sooner (i.e. the competition point was advanced) than the control colonies headed by sham-operated queens (van Honk et al., 1980). In a subsequent experiment, there was a significant effect of the mandibular gland extract or total body wash on the volume of the CA of workers as well as the rate of JH biosynthesis *in vitro* (Roseler et al., 1981). Since in these studies queen mandibular gland extracts or cuticular washes had similar inhibitory effects to those of live queens, it was suggested that the putative queen pheromone is produced in these glands and then spread over the queen body by self-grooming (Roseler and Van Honk, 1990). The mandibular gland's secretion is composed mostly of a series of 3-hydroxy acids (Hefetz et al., 1996) accompanied by lesser amounts of alcohols, 2-ketones, alkenes, alkanes, and fatty acyl esters. In an attempt to pinpoint the chemicals that may be involved in queen inhibition of worker reproduction, a re-evaluation of the queen's glands, body extracts, or washes were tested by assaying *in vitro* JH production by the CA (Bloch and Hefetz, 1999a). Using a Soxhlet washed queen (a method that thoroughly removes the lipids from the body surface) as a dummy onto which the secretion was applied, it was demonstrated that none of the glandular secretions was effective in inhibiting the activation of the CA in QL workers. This refuted the prevailing hypothesis that the mandibular glands are the sole source of such a pheromone. Notwithstanding, both body extract and body washes had an inhibitory effect on CA activation. A comparative study of cuticular washes of nest-searching queens (not reproductive yet) and egg-laying queens showed distinct cuticular profiles, suggesting that the latter possess a fertility signal (for review of the chemical ecology of bumble bees, see Ayasse and Jarau, 2014; Sramkova et al., 2008). It was further demonstrated that although egg-laying workers were distinct from the queens, their cuticular profile was more similar to egg-laying queens than non-laying workers, supporting the existence of fertility signals. While this comparative study provided correlative evidence for a queen pheromone, the direct causal experiment to test any of the compounds or the blends was not performed. A recent study tested the effect of several compounds of the above blends that were significantly more abundant in queens than in workers (Van Oystaeyen et al., 2014). Of these only pentacosane showed some activity, it acted to enhance oocyte resorption but

not to inhibit ovary activation. Since multiple studies have shown that the queen does inhibit ovary activation in pre-CP, which pentacosane seemingly failed to mimic, there are likely additional pheromones involved. Moreover, as described above, during the CP, there is also high competition among workers, and ovary resorption could have resulted from worker-worker inhibition. Finally, there is no information in the study regarding whether all workers were dissected and what proportion had developed ovaries. This is important since, as described above, not all the workers in the colony develop ovaries even during the CP.

Other exocrine glands that were chemically analysed in queens under various social conditions are Dufour's and labial glands. There were no qualitative differences in Dufour's gland secretion of pre-CP compared to CP queens (Amsalem et al., 2009). In the labial gland, there were quantitative differences in the quantities of dodecyl esters between pre-CP and CP queens (Amsalem et al., 2014a). However, since this secretion had no effect on worker reproductive inhibition (Bloch and Hefetz, 1999a), these differences may reflect a different function (see discussion in Section 6.7).

Additional studies examined the nature of the queen signal, and whether *B. terrestris* uses a queen control versus queen signal system. To address this question, nests were split into two compartments: one contained the queen brood and workers (queenright compartment, QRC), while the second contained a group of five workers (queenless compartment, QLC). Groups of five workers housed in a separate nest box served as completely QL control (Alaux et al., 2004a). In the first experiment, the compartments were separated with a double screen so that all contact between the QRC and QLC was prevented, but emitted volatiles could spread into both compartments. Under these conditions, workers in the QLC possess fully activated ovaries 7 days post-group establishment (the time needed for oocyte maturation), which was equivalent to the QL control group. Workers in the QRC laid eggs only after about 3 weeks, as expected from the timing of the CP in non-manipulated colonies. Thus, if a queen pheromone exists it is clearly not volatile. In the second experiment, the compartments were separated by a queen excluder that allowed the workers, but not the queen, to travel between compartments. To entice workers to travel between compartments, the food supply was placed in the queen-excluded compartment (QEC). The hypothesis was that if the queen coercively inhibits worker reproduction, the QEC could provide a refuge for workers wishing to escape the queen's control. However, all workers moved back and forth between compartments indicating that none 'sought refuge' from the queen.

Moreover, ovary activation of the workers was inhibited relative to workers that were completely QL, and although egg laying occurred in the QEC earlier than in the QRC, it was still near the CP and much later than egg laying by workers that were completely QL. These results lend credence to the hypothesis that workers control their own reproduction and *B. terrestris* uses a ‘queen signal’. In a subset of this experiment, five workers were confined to the QEC by glueing a bar to their thorax to prevent them from moving between compartments. These workers behaved like QL workers in that they established a dominance hierarchy among themselves and laid eggs about a week after group establishment. These results indicate that the information of the queens’ presence has to be individually acquired by each worker and cannot be transmitted between workers. It further showed that even when egg laying by workers occurs in a maladaptive timing with respect to other workers in the colony, they do not police either the egg-laying workers or their laid eggs.

4.4 Genomic factors underlying queen–worker conflict

Studies examining the genes that regulate queen–worker conflict in bumble bees have largely focused on *Krüppel-homolog 1* (*Kr-h1*). *Kr-h1* is a zinc finger transcription factor whose brain expression levels are down-regulated by queen presence in *B. terrestris* workers (Shpigler et al., 2010) and queen pheromone in honey bees (Grozinger and Robinson, 2007; Grozinger et al., 2003). The effects of the queen on *Kr-h1* expression levels are likely mediated by JH. JH titers are lower in QR versus QL workers in both bumble bees (Bloch et al., 2000a) and honey bees (Hartfelder et al., 2015; Pankiw et al., 1998). Furthermore, *Kr-h1* RNA levels in the brains and fat bodies of bumble bee (Shpigler et al., 2010, 2014) and honey bee workers (Grozinger and Robinson, 2007) are up-regulated by JH treatment. In other insects, it has been demonstrated that transcription of *Kr-h1* is activated when JH binds to the JH receptor complex (consisting of the methoprene-tolerant transcription factor and its steroid receptor co-activator, with facilitation from Hsp83), and this complex binds to the E-box motif in the promoter region of *Kr-h1* in *Locusta migratoria* (Song et al., 2014), *Bombyx mori* (Kayukawa et al., 2012), *Aedes aegypti* (Cui et al., 2014), and *Drosophila melanogaster* (He et al., 2014). Studies in honey bees suggest that *Kr-h1* expression may also be regulated by cGMP (Fussnecker and Grozinger, 2008). In other insects, *Kr-h1* mediates many of JH’s effects on developmental processes (Minakuchi et al., 2009), vitellogenesis, and oogenesis (Song et al., 2014), and also can

affect neuronal morphology (Shi et al., 2007). As a transcription factor, *Kr-h1*'s effects are due to its ability to modulate expression of other genes, including the transcription factor *E93* (Belles and Santos, 2014), though it does not appear to directly regulate expression of *vitellogenin* (*vg*) (Song et al., 2014). Recent studies in *B. impatiens* have demonstrated that *Kr-h1* expression is down-regulated in workers exposed to both uncaged queens and caged queens (which cannot approach and attack workers), suggesting that, at least in this species, *Kr-h1* expression responds to queen-produced social cues aside from aggression (Padilla, M., Amsalem, E., Hefetz, A., Grozinger, C.M., unpublished data).



5. WORKER-WORKER CONFLICT OVER REPRODUCTION

As previously described, workers compete over male production during the CP not only with the queen but also among themselves (Duchateau and Velthuis, 1988). Bumble bee workers are all morphologically capable of reproduction (Fig. 2). They have the same number of ovarioles as the queen and can produce relatively high number of eggs in a short period (a group of 10 callow QL workers that were kept for 15 days laid altogether 97 eggs, Amsalem et al., 2013a). Although workers still possess spermatheca, they do not mate and thus produce haploid males to whom they are more genetically related ($r=0.5$) than to their brothers ($r=0.25$) or nephews ($r=0.375$). However, there are several constraints to the time-window available for worker reproduction. Seasonal limitations, such as the availability of resources needed to rear brood, predict that only male eggs that are laid early enough in the season will successfully develop into adulthood as well as being synchronized with the mating period of the gynes. Inclusive fitness constraints predict that workers will not start reproducing before gynes are produced by the colony (Alaux et al., 2006). Furthermore, both the queen and other workers will destroy worker-laid eggs: while queen-laid eggs are only rarely eaten during the CP (Zanette et al., 2012). Consequently, only a small portion of the eggs laid by workers will successfully develop into adult males (Alaux et al., 2004b; Brown et al., 2003a; Owen and Plowright, 1982; Paxton et al., 2001). Thus, although all workers are capable of reproduction, there is a clear advantage for workers who reproduce as early as possible, predicting a high competition between workers over male production even before the competition actually starts.

The competition among workers is largely regulated by aggression, dominance, and hierarchy formation. QL conditions and specifically small,

same age groups of workers provide an excellent opportunity to (1) test dyadic relationships in more detail, (2) understand how reproduction is regulated, (3) unravel the role of aggression in mediating hierarchy formation, (4) explore how dominance behaviour may act as a driving force for more advanced social features such as division of labour, and (5) the study of QL groups (in particular larger groups) can also be extrapolated to understand events occurring during the CP in QR colonies, and thus, provide a powerful tool to examine social behaviour in bumble bees.

5.1 Rates of worker reproduction

Worker reproduction is widespread in Hymenoptera and is particularly common in bumble bees (Bourke, 1988). In *B. terrestris*, in line with the abovementioned theoretical predictions, it is limited and only around 40% of workers eventually lay eggs either during the CP or under QL conditions. Despite different approaches for estimating worker reproduction in the literature, similar results were obtained for entire colonies or small QL groups. For example, Alaux et al. (2004b) found 64% of the workers with ovaries in stage 3–4 (terminal oocyte size >1.27 mm) and 38% egg-layers in colonies. Duchateau and Velthuis (1989) found 36% of workers with ovaries in stage 4–5 (terminal oocyte size >2.43 mm) in colonies. Amsalem et al. (2009) found 42% of workers with terminal oocyte size larger than 1 mm in either QL or CP colonies, and Amsalem and Hefetz (2011) found 45% with terminal oocyte >0.5 mm in 5-day-old workers from same-aged QL groups containing 10 workers.

5.2 Worker reproductive hierarchy

Hierarchy among workers can be defined in terms of either aggressive behaviour or reproductive output. The two hierarchies are highly correlated but not necessarily identical, suggesting that they are regulated by different pathways that interact with each other. In QL groups, the most aggressive worker is commonly the most reproductive, with a 94% match between aggression and reproduction in paired bees (Amsalem and Hefetz, 2010) and an 80% match in three-worker groups (Amsalem et al., 2014c). Supporting the disparate regulation of behavioural and reproductive dominances is the finding that ovariectomized workers, although unable to lay eggs and do not construct egg cells, perform the same ritualistic dominance behaviours as non-operated dominant workers (Doorn van, 1989).

Consistent with worker–worker competition, dominant workers are able to inhibit ovarian activation in nestmates (Amsalem et al., 2013a; Bloch and Hefetz, 1999b; Cnaani et al., 2007; van Honk and Hogeweg, 1981). However, ovarian suppression is not absolute, and in larger groups, it is less effective; so instead of one worker dominating reproduction, a few workers will share egg laying (Amsalem and Hefetz, 2011). *B. impatiens*, given enough time subordinate workers in small groups (even in paired bees), can activate their ovaries and lay eggs. Moreover, a dominant worker that had many sons had also many nephews, indicating that a very fertile worker stimulates her group-mates to lay more eggs (Sibbald and Plowright, 2013, 2014).

Interestingly, both ovary activation in dominant workers and suppression in subordinate workers depend on physical contact: workers kept in pairs but separated by a single or a double mesh show low ovarian activation, but when given the opportunity to interact by removing the mesh, aggression ensues resulting in a clear reproductive hierarchy formation (Amsalem and Hefetz, 2010). Furthermore, workers seem to require these aggressive social stimuli to initiate reproduction as ovarian activation and egg laying are greatly delayed in isolated workers (Amsalem and Hefetz, 2010; Amsalem et al., 2009; Duchateau and Velthuis, 1989). Larger groups enhance ovarian activation in group-mates compared to smaller groups (Duchateau and Velthuis, 1989), but the effect is rank dependent: while the α -worker rapidly activates her ovaries to the maximum irrespective to the group size, workers of lower ranks in large groups activate their ovaries faster than same-rank workers in smaller groups (Amsalem and Hefetz, 2011), emphasizing the importance of social interactions for reproduction.

5.3 Aggression and hierarchy formation

Although overt worker–worker aggression is observed primarily in the CP, it is likely that worker reproductive and behavioural hierarchies are formed earlier in the colony cycle. Even during the pre-CP, a distinct group comprising around 40% of the workers tends to interact more frequently among themselves (and with the queen) than with the other workers, forming an ‘elite worker group’ (Doorn van and Heringa, 1986; van Honk and Hogeweg, 1981). This designation describes workers that have activated ovaries (though they do not appear to lay eggs), reside near the queen, and later, during the CP, become egg-layers and display the most aggressive behaviours towards other elite workers as well as the queen (Doorn van and

Heringa, 1986). The queen may maintain her α -position for a limited period (Doorn van and Heringa, 1986), but during the CP, she often loses her superiority and can die or be killed by the workers (Bourke, 1994). Upon queen loss, aggression and the formation of dominance hierarchy among workers also occur in full-fledged colonies (Amsalem et al., 2013a; Doorn van, 1986, 1989; Doorn van and Heringa, 1986; van Honk and Hogeweg, 1981; van Honk et al., 1981).

Dyadic interactions among workers is the foremost method used to describe the formation and maintenance of the dominance hierarchy. These interactions were incorporated into mathematical models to describe the complex relationship between individuals in entire colonies or large groups (Amsalem et al., 2013a; Hogeweg and Hesper, 1983; van Honk and Hogeweg, 1981) or used to quantify aggression in small QL groups in one of two indices: the ‘aggression index’ and the ‘dominance index’. The aggression index scores three types of aggressive behaviours. The two that are considered threatening behaviours are *Humming*, defined as short bursts of wing vibrations directed at another bee, and *Darting*, defined as a sudden and directed movement towards another bee without making actual contact. The third behaviour is an escalated *Attack*, defined as physical contact resulting in biting, pushing, dragging, struggling, or attempted stinging. The aggression index is the sum of these three behaviours. Workers that score high on the aggression index possess higher level of ovarian activation, higher JH levels, higher *vg* expression levels in both fat body and head, and produced less (or no) sterility signal in their Dufour’s gland (Amsalem and Hefetz, 2010, 2011; Amsalem et al., 2014b,c). When aggression is scored solely based on the number of physical attacks, it is strongly correlated with the number of observed oviposition events per bee (Doorn van and Heringa, 1986) and with ecdysteroid titers in the ovaries and hemolymph (Geva et al., 2005). For the dominance index, aggression is scored based on the number of advances versus retreats each individual bee displays in each dyadic encounter (Doorn van and Heringa, 1986; van Honk and Hogeweg, 1981). The dominance index is defined as $[1 - (\text{retreats}/\text{total encounters})]$ (Bloch et al., 1996). This dominance index yielded correlations with *Kr-h1* expression levels, a gene that is regulated by JH (Shpigler et al., 2010).

Levels of aggression change dynamically during the course of dominance hierarchy establishment in QL worker groups. There is a peak of aggression during the first days post-group establishment that declines until there is little aggression apparent once the hierarchy is established. Similar results were obtained using either the aggression index or the dominance index

(Amsalem and Hefetz, 2010, 2011; Bloch et al., 1996). Aggression is also highly variable among both individuals and groups, with some groups showing high aggression levels while others show none. Since aggression starts very early, it seemingly precedes both ovarian activation and most of the hormonal changes in workers (Amsalem and Hefetz, 2010). In most cases, a single bee performs most of the antagonistic behaviours (Amsalem and Hefetz, 2011). A simulation model based on *B. terrestris* biology aiming to test the interplay between aggression and reproduction suggested that aggression and the outcome of previous dyadic encounters are the major determinants of ovarian activation and not vice versa (Amsalem et al., 2013a). Workers may gain a head start in the competition by fortuitously winning their first encounters, or due to variation in their innate aggressive tendencies, or because of any other physiological and morphological differences between them.

5.4 Chemical communication versus dominance behaviour

The decreased aggression observed after dominance hierarchy establishment in QL worker groups may indicate that subordinate workers have succumbed to their role, and/or social signals, such as pheromones, have replaced aggression to maintain the social structure while minimizing the cost of aggression. Indeed, there are several lines of evidence that indicate that dominant workers, subordinate workers, and brood produce a variety of pheromone signals (reviewed in Ayasse and Jarau, 2014). Dominant workers can be discriminated from sterile workers by several chemical markers that are associated with either their fecundity, aggressiveness, or both. Group-specific odour bouquets (cuticular and gland extracts as well as headspace volatiles) are found in dominant versus subordinate workers of *B. hypnorum* (Ayasse et al., 1995). In *B. terrestris*, cuticular hydrocarbons were found to steadily increase in workers with activated ovaries during the CP (Sramkova et al., 2008) and cuticular lipids were associated with reproductive status (Blacher et al., 2013a). The latter led to higher rejection and aggression towards fertile versus sterile workers by non-nestmate workers (Blacher et al., 2013a). Furthermore, the Dufour's gland secretion in *B. terrestris* shows a caste- and reproductive-state-specific composition. While the queen's secretion is composed of alkanes and alkenes, workers possess an additional series of octyl esters. These octyl esters are also negatively correlated with ovarian activation, declining to nearly undetectable levels in workers with activated ovaries (Amsalem et al., 2009). When placed

in pairs, aggression towards subordinates was negatively correlated with the proportion of octyl esters they produced (Amsalem and Hefetz, 2010), suggesting these esters signal sterility to dominant workers and queens, rendering subordinate workers safe from attacks. A similar reproductive-state- and caste-related secretion composition also exists in the labial glands of *B. terrestris*. Infertile individuals (both workers and virgin queens) produced a series of dodecyl esters and several other hydrocarbons, the amount of which is negatively correlated with ovarian activation (Amsalem et al., 2014a).

It is fascinating to consider the evolutionary mechanisms underlying the phenomenon of signalling sterility. Although advertising sterility or fertility are two sides of the same coin, it is intriguing that *B. terrestris* workers actively invest in producing sterility-specific compounds. While the advantage of evolving a fertility signal is evident, how advertising sterility confers an advantage to the signalling bees is not intuitively obvious. We suggest that in small-size eusocial colonies that exhibit high aggression towards workers with developed ovaries and where workers may have an equivalent reproductive potential, as in the case of *B. terrestris*, signalling of sterility confers a high advantage to all partners in reducing unnecessary harassment of bees that are not in competition for reproduction and thus enhances social harmony in the seemingly chaotic CP. Indeed, the sterility signal along with a possible fertility signal in workers (Sramkova et al., 2008) provides a means for fine-tuning the division of labour in CP colonies, enabling the successful rearing of reproductives (males and gynes) by allowing continuous provision by unintimidated foragers. Unfortunately, the glandular source of the observed fertility signal in *B. terrestris* is still elusive, preventing any investigation into the mechanisms underlying the evolution of such dual pheromonal regulation. Similarly, it is interesting that both the Dufour's and labial gland secretions appear to serve the same function. We can attribute such redundancy for fine-tuning the delicate regulation of division of labour. However, it is also likely that this is the result of ongoing queen-worker and worker-worker social conflicts and represents an escalated means to resolve these conflicts (i.e. an arms race). As one party evolve means (e.g. pheromones) to outcompete its competitors, the latter evolve means to neutralize them (e.g. developing insensitivity to the means), leading to the evolution of new means (new pheromones) and so forth (Katzav-Gozansky, 2006).

Dominance, reproductive status, and aggression may be also modulated by chemicals produced by developing brood. When pairs of QL *B. impatiens* workers are exposed to developing brood, they exhibit reduced frequency

and duration of aggressive behaviours (Sibbald and Plowright, 2014), suggesting that developing brood may trigger rearing behaviours and therefore disrupt formation of dominance hierarchies. Developing brood may signal to workers that the queen is still functioning, and it would be selectively advantageous for them to continue to rear sisters, or that the reproductive dominance hierarchy among QL workers has already been established.

5.5 Hormonal regulation of reproduction and dominance

Hormones play an important role in regulating reproduction and dominance behaviour in many insects (Hartfelder, 2000; Nijhout, 1994). As described above, JH in particular was extensively studied in bumble bees, and its role as a gonadotropin is well established using both correlative (Bloch et al., 1996; Roseler, 1977; Roseler and Roseler, 1978, 1988) and direct evidence. Both surgical removal of the CA (allatectomy) (Shpigler et al., 2014) and treatment with precocene-I (a JH inhibitor) (Amsalem et al., 2014c) reduce JH levels and consequently ovarian activation in QL workers, and the latter was rescued by a topical application of JH-III. However, it was long debated if JH also affects aggressive behaviour in bumble bees as it does in primitively eusocial wasps (Barth et al., 1975; Roseler et al., 1980, 1984). Studies found positive correlations between JH levels as well as the expression levels of *Kr-h1*, a gene that is regulated by JH and dominance behaviour (Amsalem et al., 2014c; Bloch et al., 1996; Shpigler et al., 2010). However, these correlations could be mediated by ovarian activation, since aggression precedes ovarian activation in QL workers and is highly correlated with it in advanced age (Amsalem and Hefetz, 2010). Direct manipulation in which a random worker was treated with JH in four-worker groups failed to show an increase in the treated worker's chances to gain dominance over her group-mates (Doorn van, 1989), but these experiments are still inconclusive since JH-I was used rather than JH-III, the natural hormone in *B. terrestris* (Bloch et al., 2000a). In a slightly different experimental design where callow workers were introduced to peer (two workers of the same age and size) or established groups (two older workers), the introduced workers did not show an increase in aggression as a function of the JH-III treatment, irrespective of the social set-up they were exposed to (Amsalem et al., 2014b). Furthermore, although precocene-I decreases aggression in workers, a single treatment with JH did not reverse this effect (Amsalem et al., 2014c). Thus, all the evidence so far supports a model in which a change in aggression leads to a change in JH levels, but JH does not directly

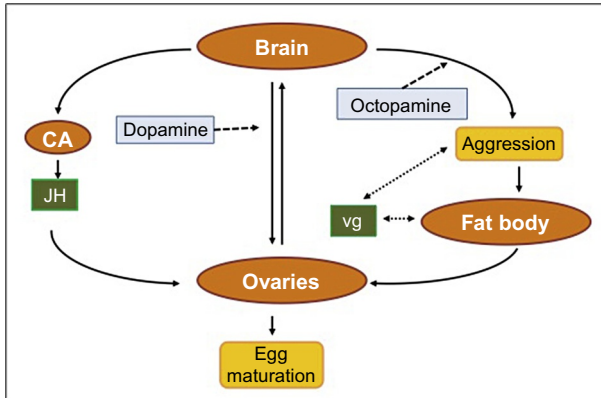


Figure 3 Simplified schematic representation of the hypothesized pathways regulating aggression and ovarian activation in *B. terrestris* workers. Signals from the brain stimulate the corpora allata (CA) to produce juvenile hormone (JH). High JH titers stimulate ovarian activation. Independently, a second set of signals from the brain (likely mediated by octopamine signalling pathways) triggers aggression. Aggression is correlated with high expression levels of vitellogenin (vg) in the fat bodies, though it is unknown whether high vg triggers aggression or vice versa. High levels of aggression and/or vg lead to ovarian activation and to a change in JH via a possible positive feedback between the ovaries and the brain. Egg maturation and egg laying are also possibly mediated by a positive feedback between the ovaries and the brain via dopamine signalling pathways.

regulate aggression (Fig. 3). Since sterility in *B. terrestris* is also tightly related to the division of labour among workers (Amsalem et al., 2013b; Roseler and Van Honk, 1990), JH was tested for its possible role in regulating task. However, unlike the honey bee, JH does not play a role in regulating division of labour in bumble bees, with foragers and nurses showing similar JH titers (Cameron and Robinson, 1990; Doorn van, 1986).

Both the behavioural and physiological evidence indicate high regulation by brain factor, most probably biogenic amines (BA). However, we have little knowledge on how these affect both queen and worker behaviour. Quantification of three major brain BA, dopamine, serotonin, and octopamine revealed that there were differences between virgin and mated, egg-laying queens (Bloch et al., 2000c). Workers on the other hand showed differences with respect to both reproductive and behavioural dominance. Reproductive workers, irrespective of their dominance status, showed higher dopamine levels in the last stages of oocyte development, while dominant workers, irrespective of their reproductive state, showed higher levels of octopamine. Serotonin levels were similar in all workers. While these

findings indicate the differential roles of dopamine and octopamine in the reproductive competition among workers, it awaits more causative experimentation.

Vg in most insects is the major yolk protein, and accordingly, the expression levels of its gene (*vg*) are regulated by JH (Chen and Hillen, 1983; Engelmann, 1979; Hagedorn and Kunkel, 1979). In social insects (e.g. the honey bee), Vg has assumed additional regulatory roles (Amdam et al., 2004, 2006). This may be also the case in *B. terrestris*. The level of ovarian activation in *B. terrestris* workers showed only partial correlations with *vg* levels (Amsalem et al., 2014b), and *vg* expression levels were found to correlate with aggressiveness in QL workers that were kept in small controlled groups and were too young to show any significant differences in ovarian activation. Thus, *vg* expression levels in *B. terrestris* workers appear to primarily correlate with aggression and only secondary with reproduction (Fig. 3). Accordingly, the interrelation between JH and *vg* did not follow the common pattern in most insects, where JH directly regulates the levels of *vg*. Although there is an overall positive correlation between JH levels and vitellogenin (mRNA expression and protein level) in *B. terrestris* QL workers (Shpigler et al., 2014), a direct manipulation of JH in QL workers did not affect the *vg* expression levels (Amsalem et al., 2014b). While this suggests that JH and *vg* are uncoupled in *B. terrestris* and is consistent with the hypothesis that *Bombus* represents an intermediate stage in the evolution of eusociality, more genomic data are needed to fully explore this hypothesis.

Ecdysteroids also appear to regulate dominance behaviour in workers. In QL groups, high social status is associated with high ecdysteroid titers but is limited to a certain age and does not correlate with ovarian activation (Bloch et al., 2000b). In another study, ecdysteroid levels in both the ovaries and hemolymph positively correlated with different stages of ovarian activation, albeit only weakly with the terminal oocyte size. Likewise, ecdysteroid titers were correlated with overt aggression, but not with threatening behaviours (Geva et al., 2005). Brood presence and group size also influenced the level of ecdysteroids in QL workers (Geva et al., 2005). Thus, ecdysteroids titers are clearly sensitive to social interactions, but its role in mediating reproductive dominance remains to be deciphered.

5.6 Genomic mechanisms underlying worker–worker conflict

Genomic approaches have provided insights into the mechanisms mediating worker–worker conflict over reproduction in QL *B. terrestris* worker groups. Dominant workers have significantly higher expression levels of *Kr-h1* in

their brains than subordinate workers, which mirrors the higher levels of *Kr-h1* found in the brains of QL versus QR workers (Shpigler et al., 2010). Allatectomy of young QL bumble bee workers results in decreased fat body expression levels of *Kr-h1* and *vg*, along with a decrease in JH titers and ovary activation in 7-day-old bees. Exogenous treatment of intact (non-operated) QL workers with a single JH dose increases *Kr-h1* expression (Shpigler et al., 2010) but does not increase *vg* levels (Amsalem et al., 2014b), which is consistent with *Kr-h1* functioning upstream of *vg*. These data, coupled with the studies of JH, aggression, ovary activation (see above), suggest that in bumble bees *vg* levels are either indirectly regulated by JH (likely through *Kr-h1*) or co-regulated by another pathway that is associated with aggression (see Fig. 3).

DNA methylation may also play a role in worker–worker conflict in *B. terrestris*. Using methylation-sensitive amplified fragment length polymorphism methodology, it was found that genome-wide DNA methylation patterns differ significantly between QL reproductive workers and both QL and QR non-reproductive workers (Amarasinghe et al., 2014); however, this study needs to be repeated using more accurate sequencing-based methods. Treatment of young (but not mature) QL worker bees with an inhibitor of DNA methylation resulted in increased aggression and ovary activation levels (Amarasinghe et al., 2014). However, the changes in methylation levels in the loci examined were complex—the majority of loci showed no change, and an equivalent number of loci (9 and 10, respectively) were hyper- or hypo-methylated in treated bees versus controls, and thus it is not clear if DNA methylation actually has a functional role in this process. The function of DNA methylation in insects is still under investigation, but it may primarily modulate splicing rather than transcription (Flores et al., 2012; Li-Byarlay et al., 2013; but see Wang et al., 2013). Interestingly, alternative splicing of the transcription factor *gemini* can lead to worker ovary activation in honey bees (Jarosch et al., 2011). Other studies have indicated that decreasing methylation levels biases developing honey bee larvae to queen rather than worker development (Kucharski et al., 2008), suggesting that methylation plays a general role in regulating reproductive potential in bees. For a comprehensive review of the effect of epigenetics on social behaviour, see Glastad et al. (2015).

5.7 Factors affecting dominance and reproduction in workers

Factors determining dominance rank and reproductive potential of workers largely depend on morphological and physiological characteristics such as

age, body size, and task, but also on external factors including colony demography and spatial organization of workers in the nest. In the following paragraphs, we will briefly present recent findings relevant to each of these factors and their effects on worker reproduction.

Internal factors – Age has a strong effect on the likelihood of a worker to achieve dominance, though this depends on the social context. Under QL conditions, when workers of similar sizes but of different ages are housed together, reproduction is often dominated by the older worker (Amsalem et al., 2014b; Doorn van, 1989). Even age difference of as little as 5 h can increase the probability of a worker to become dominant (Doorn van, 1986). In QR colonies during the CP, the first egg-layers are often the oldest workers who emerged in the first or second brood. However, once the competition starts, worker age seems to no longer play an important role in the probability of workers to become egg-layers (Doorn van and Heringa, 1986; van Honk et al., 1981).

Bumble bees are often typified by size polymorphism among workers, and the fact that aggression is a major means by which workers may gain reproductive dominance suggests that size may have an impact on worker probability to become dominant (for discussion on the factors affecting worker size polymorphism in bumble bees, see Couvillon and Dornhaus, 2010; Couvillon et al., 2010; Duchateau and Velthuis, 1988; Owen, 1988, 1989; Shpigler et al., 2013; Sutcliffe and Plowright, 1988). Size seems to have a small effect mainly under QL conditions: in small groups of four workers of unequal body size, there was a positive correlation between body size and dominance rank, but the probability of the largest bee in each group to become dominant was not raised significantly (Doorn van, 1989). Under QR conditions, worker size was only important for the workers that emerged from the first batch of eggs (van Honk et al., 1981), but otherwise body size did not seem to play an important role in determining which worker will be an egg-layer (Duchateau and Velthuis, 1989).

Task allocation among workers in a QR colony may also affect the probability to become dominant. Although previous studies suggested that worker division of labour in bumble bees is more flexible than many other social insects (Doorn van and Heringa, 1986; Jandt and Dornhaus, 2009; O'Donnell et al., 2000; Yerushalmi et al., 2006), bumble bee workers do exhibit task specialization (Doorn van, 1986) that is loosely dependent on adult size (alloethism) rather than on worker age (polyethism) (Cameron, 1989; Cameron and Robinson, 1990; Goulson et al., 2002). In general,

smaller workers tend to perform nurse-like tasks, while larger workers tend to be foragers but age at task varies widely between individuals (for detailed discussion on the effect of worker size and age on task allocation, see [Amsalem et al., 2013b](#); [Cameron, 1989](#); [Cnaani and Hefetz, 1994](#); [Doorn van, 1986](#); [Goulson et al., 2002](#); [O'Donnell et al., 2000](#); [Spaethe and Chittka, 2003](#); [Spaethe and Weidenmuller, 2002](#); [Worden et al., 2005](#); [Yerushalmi et al., 2006](#)).

In *B. terrestris*, worker task strongly affects the likelihood that workers become reproductively dominant. A non-forager bee is twice as likely to become an egg-layer during the CP than a forager bee, and workers that have been observed to forage prior to the day they were killed had lower ovarian activation compared to workers that did not forage ([Duchateau and Velthuis, 1989](#)). Most likely, foragers simply have much less energy to invest into ovarian activation than nurse bees ([Foster et al., 2004](#)). *B. terrestris* foragers have also been found to produce larger amounts of octyl esters in their Dufour's gland (the previously discussed sterility signal) compared to nurses, and these quantities correlate with their number of foraging flights ([Amsalem et al., 2013b](#)).

As described previously, aggression is a decisive factor in establishing dominance hierarchy. When workers are kept in small controlled groups, they exhibit aggression relatively early in a predictable timetable. This aggression precedes most of the physiological changes in workers and is strikingly diverse even between workers who share the same physiological, morphological, and social conditions. It is therefore likely that aggression will be regulated by intrinsic factors, possibly genetic, that determine which bee will be more aggressive and thus will gain dominance and lay eggs.

External factors – The variability in worker aggression and reproduction can also be partially explained by external factors such as the population composition and in-nest organization, which may affect not only the chances of workers to be egg-layers but also determine their final size and role. *B. impatiens* larvae at the nest centre are better fed and grow larger, while those in the periphery of the nest are fed less and develop into smaller adults ([Couvillon and Dornhaus, 2009](#)). The smaller adult workers, on the other hand, tend to reside near the centre of the nest and perform nurse-like tasks such as larval feeding, while the larger workers tend to reside on the nest edges and are more likely to be foragers ([Jandt and Dornhaus, 2009](#)). Thus, it is likely that in *B. impatiens*, larvae reared in the centre of the nest are more likely to become non-reproductively active workers.

Examination of the effects of brood presence on worker reproduction showed ambiguous results: ovarian activation was not significantly different when workers were exposed to larvae (Duchateau and Velthuis, 1989) or kept in pairs with or without brood (Sibbald and Plowright, 2013). However, in the presence of brood, ovarian mass was higher (Sibbald and Plowright, 2014). The presence of gynes is highly correlated with worker reproduction in CP colonies (Cnaani et al., 2000a; Duchateau and Velthuis, 1988; Lopez-Vaamonde et al., 2007).

Another important social context affecting the probabilities of workers to reproduce is group size, via its effect on the cost of aggression versus gain in reproduction. The frequency of aggressive behaviour in the α -workers increased proportionally with group size (Amsalem and Hefetz, 2011), suggesting that the α -worker adjusts her level of behavioural dominance to control groups of different size. However, the ability of the α -worker to control her group-mate's reproduction is not unlimited. In larger groups, although the α -worker seems to attain her maximal aggression level, it is still insufficient because other members of the group possess activated ovaries. Therefore, there seems to be a trade-off between the cost of being aggressive and the gain of being the only reproductive, which imposes limits on aggressiveness. Nonetheless, the findings that both aggressive and aggressed workers show higher levels of *vg* expression and ovarian activation compared to passive workers (Amsalem et al., 2014b) raise the possibility that increased social interactions enhance ovarian activation.

5.8 Drifting bees: Fortuitous error or intra-specific parasitism?

Drifting of workers from their natal colony to a host colony may be the consequence of navigation errors coupled with mis-identification of their own nest location. However, molecular studies revealed that such drifter might lay eggs and contribute to male production of the host colony. Thus, drifting can be considered as an alternative reproductive strategy and social parasitism. Drifting and subsequent egg-laying by non-natal workers has been recorded in several bumble bee species. In wild and laboratory-reared, free-foraging *B. terrestris* colonies, drifting was estimated at 2–3%, and drifting bees produced twice as many eggs as resident workers (Lopez-Vaamonde et al., 2004; O'Connor et al., 2013; Zanette et al., 2014). In a study of *B. deuteronymus*, 3 out of 11 colonies were invaded by non-natal workers that produced 19% of the males, compared to 17% of male production by the resident workers (Takahashi et al., 2010). In managed bumble

bee colonies, drifting was reported to be more common: in *B. impatiens* and *B. occidentalis*, 28% of the bees drifted (Birmingham and Winston, 2004; Birmingham et al., 2004) and the numbers were higher for managed *B. terrestris* colonies, reaching up to 50% when the colonies were vertically stacked (Lefebvre and Pierre, 2007). Thus, drifting may be greatly influenced by the geographical distance between colonies, which in managed bees tend to be much smaller compared to natural conditions. Interestingly, 60% of a cohort of marked reproductive or subordinate *B. terrestris* workers visited other nests, with the fertile workers drifting at higher rate compared to the unfertile (Blacher et al., 2013b).

The question of whether drifting is a result of fortuitous error or social parasitism still remains to be determined. Increased drifting as a function of nest proximity lends credence to the hypothesis that drifting is a result of navigation errors. Negating this hypothesis is the fact that in nature, nests are more dispersed and there is low probability of navigation error, and yet drifting is still observed in wild colonies. Furthermore, reproductive workers are more likely to drift, though reproductive workers are also more likely to refrain from foraging, suggesting that when they exit their natal nest it is specifically to search for another bumble bee colony. The question of why drifter eggs are not destroyed by the resident bees also remains unanswered. During the CP, oophagy by the resident bees is mostly directed to other workers' eggs but not those of the queen (Zanette et al., 2012), raising the possibility that drifters somehow mask their eggs to become indistinguishable from queen eggs.

5.9 Extrapolating from QL groups to whole QR colonies

The social structure and division of labour in *B. terrestris* QR colonies is maintained not only during the eusocial phase but also under the chaotic conditions of the CP. Moreover, under QL conditions, lethal aggression is rare and larvae are still fed by subordinate helpers, and many tasks are performed continuously (although some of them decreased as the colony ages) (Foster et al., 2004; O'Donnell et al., 2000). We propose that the study of QL groups can be extrapolated to understand events occurring during the eusocial phase of bumble bee colonies.

While small controlled groups of three workers provide insight into the mechanisms regulating hierarchy and reproduction among nest-mates, they can hardly simulate whole colonies. For example, patterns of reproduction and pheromone production in 3- and 5-worker groups

were clearly different from those of 10-worker group or whole colonies (Amsalem and Hefetz, 2011). However, as previously mentioned, increase in group size is correlated with elevated aggression and lower reproductive suppression (Amsalem and Hefetz, 2011), providing an insight into the mechanisms by which the queen may inhibit worker reproduction during the pre-CP when worker reproduction starts due to the queen's inability to physically inhibit worker oviposition. These findings emphasize the role of aggression in shaping and maintaining the social structure.

Task allocation correlates in workers may also resemble the behavioural transition of queens from the solitary to the eusocial phases. Foraging, being risky, is, in most social insects, the last task that a worker performs in her life. In contrast, in bumble bees, foraging is usually the first task workers perform. Workers start foraging at the age of 2–3 days and when older they may switch to in-nest tasks. Foragers are typically sterile and non-aggressive, contrary to the typically old and aggressive, in-nest house bees. Similarly, post-diapause queens forage during the solitary phase, but switch into in-nest tasks with the establishment of the new colony and egg laying, suggesting that there might be similar physiological processes involved in these transitions.



6. LARVAL DEVELOPMENT AND MECHANISMS UNDERLYING CASTE DETERMINATION

One of the features that characterize insect eusociality is clear bimodal size distribution (queens are much larger than workers with almost no overlap) and/or distinct morphology between castes. Depending on the species, size differences between bumble bee queens and workers may be continuous (as exemplified in the North American species *Bombus nevadensis*, *Bombus borealis*, and *Bombus perplexus* and European species *B. hypnorum*) or bimodal (in the North American species *Bombus terricola*, *B. ternarius*, and *Bombus rufocinctus* and European species *B. terrestris*) (Plowright and Jay, 1968) (figure 3.1 in Goulson, 2010; Roseler, 1989). The factors affecting worker and queen sizes may be numerous and differ according to the species, including quantitatively differential feeding, worker/queen-derived factors, or the consequences of colony demographic development (Goulson, 2010 and references therein). Here, we will focus the discussion mostly on *B. terrestris* for which there is voluminous information as to the factors affecting caste

determination, that is, the bifurcation in diploid female larvae developmental pathways to either worker or queen.

6.1 Larval development

Larval developmental time in *B. terrestris* from hatching to end of the feeding period is about 18 days for queens and 15 days for workers. There is also a difference in duration from the pre-pupal stage to adulthood, again longer for queens than for workers (~ 13 and ~ 10 for queens and workers, respectively Cnaani et al., 2000b). Similar differences between queens and worker developmental duration were also reported for *B. impatiens* (Cnaani et al., 2002). The larvae, irrespective of caste, undergo four larval instars as determined by the distribution of the width of the larval head capsule (the head capsule is the only sclerotized part of the larvae which does not change in size during the instar and therefore represents larval size between instars, irrespective of the increase in weight and other body parts). There is a complete size overlap between worker- and queen-destined larval size distributions during the first two instars, but the distributions begin to separate in the third instar until finally by the fourth instar there is no overlap in sizes between the castes (Cnaani, 1998; Roseler, 1989). Accordingly, the final larval weight for queens (1.3 g) is much greater than workers (0.3 g). There are, however, no differences in growth rate between castes, indicating that the greater size and weight of queen larvae is due to prolonged developmental time rather than speed of growth. The duration of each of the last three instars in queens is longer than those in workers (Cnaani et al., 1997).

6.2 The critical period for caste determination

The critical period for caste determination is during the second larval instar, when the larvae are approximately 5 days old. To elucidate the critical age for queen determination, larvae at different ages were transferred from young colonies (worker-development conducive conditions) to QL groups (queen development conducive conditions). Almost all (80–100%) of the 1- to 4-day-old larvae developed into queens, whereas almost all (80–100%) of the 6- to 8-day-old larvae developed into workers. Among the 5-day-old larvae transferred, about half developed into queens and half into workers (Cnaani et al., 2000b). To reveal whether and when the queen presence can reverse larval determination, larvae reared under queen-conducive conditions (housed with 12 old workers that have reared queens before) were transferred to worker-conducive conditions (young colonies

that rear almost exclusively workers). Among the 5-day-old queen–destined larvae, only 30% developed into queens, compared to 60% for such 6-day-old larvae (Cnaani, 1998).

Earlier studies with *B. terrestris* gave different result (Roseler, 1970, 1976); the critical period for queen determination was found to be earlier (3.5 days as opposed to 5 days) and an attempt to reverse queen development to worker development under QR conditions resulted in a queen size bee, although they were physiologically and behaviourally worker-like. We can attribute this difference to differences in the method for determining the critical period as well as possible between-population differences: Central Europe versus the much more southern population in Israel.

6.3 Hormonal and genomic regulation of caste determination

In addition to their roles in regulating behaviour and reproduction in adult bumble bees, JH and ecdysteroids also regulate larval development and caste differentiation in bumble bees; indeed, these are the major hormones regulating larval development in most insects (Hartfelder et al., 2015; Nijhout and Wheeler, 1982; Wheeler, 1986). In social insects, the endocrine system is presumably responsive to the nutritional state of the larvae as well as the social conditions under which they grow. A detailed study of JH biosynthesis during larval development in *B. terrestris* was performed by Cnaani et al. (1997). To neutralize the differences in instar duration and larval weight between queen and workers, JH biosynthesis rates were compared between queen and worker–destined larvae of the same physiological state (for definition of physiological state, see Cnaani et al., 1997). In worker–destined larvae, JH biosynthesis rates were consistently low throughout the entire larval development, but those of queens showed two distinct peaks. The first peak started in the middle of the first instar and declined to worker levels by the end of the instar. The second peak started from the middle of the second instar and declined to worker levels by the end of the third instar and through the fourth instar. A subsequent study also measured the actual JH titers in the blood during larval development revealing high correlation between the two methods of JH measurements (Cnaani et al., 2000a). Previous studies that measured JH levels were limited to the pre-pupal stage and gave similar differences between queens and workers (Strambi et al., 1984).

The first JH peak roughly coincides with the critical period for caste determination, and can be considered as queen development commitment

peak, although experimental evidence is lacking. The second larger JH peak occurs after the larva has committed to queen development. It is assumed that these high JH levels delay moulting to the next instar, resulting in the prolonged instars observed in queen-destined larvae, and consequently greater growth. In Lepidoptera, JH is known to inhibit the prothoracicotropic hormone and with that the events cascade leading to moulting (Riddiford, 1981).

The role of increasing JH titers in triggering the onset of queen-destined development was confirmed by examining the effects of external application of JH (Bortolotti et al., 2001). JH applied to workers reared under conditions that are conducive to worker development in the first or second instar (attempting to mimic the two JH peaks in queens as determined by Bortolotti et al., 2001; Cnaani et al., 1997) resulted mostly in queen development. Interestingly, larvae of the second brood were more responsive to the JH treatment than those of the third brood, and among the former, treatments of second instar were more effective than treatments of the first instar larvae in turning worker-destined into queen-destined larvae.

Ecdysteroid is another hormone that shows differential titers between queen- and worker-destined larvae. For *B. terrestris*, two separate studies provided an almost complete picture of ecdysteroid titers during larval development (Hartfelder et al., 2000; Strambi et al., 1984). There are two clear peaks roughly in the middle of the second and third instars. Though generally queens demonstrated higher titers, this difference is significant only during the second instar. These peaks seem to be equivalent to the general pre-moult increase in ecdysteroid in holometabolous insects. Likewise, during the fourth instar, there is a small peak that could be analogous to the prepupal commitment peak that was described for the hawkmoth *Manduca sexta* (Riddiford, 1981). This ecdysteroid peak is delayed in queens until they reached a greater weight compared to workers, consistent with the prolonged instar duration, suggesting that its function in committing the larvae to metamorphose. Finally, there is a large ecdysteroid peak in the pre-pupal stage, probably inducing pupation. This peak in queens is delayed until they reach the appropriate weight for pupation, again consistent with the size differences between queens and workers.

Comparing the differences between queen- and worker-destined larvae in JH and ecdysteroid titers revealed that these differences are synchronized during larval development. Thus, although JH is traditionally considered as the regulator of caste determination, whereas ecdysteroids regulate moulting, their synchronization indicates that both may be involved in caste

determination and further suggest that they interact during this process (Hartfelder et al., 2000).

Thus far, only one study has sought to identify genes involved in caste determination in *B. terrestris*. Pereboom et al. (2005) used subtractive hybridization to identify 12 genes differentially expressed between early and late instars of queen- and worker-destined larvae. Interestingly, it seems that temporal pattern of gene expression, rather than expression *per se*, is important for driving caste differentiation: for example, genes expressed at high levels in early-instar queens versus workers were subsequently expressed at high levels in late-instar workers versus queens. Since only a few genes were identified, it is difficult to determine what general processes underlie caste determination, but several identified genes are involved in nutrient storage and metabolism. Four of the genes (*hexamerin*, *ATP-synthase beta subunit*, *Cytochrome Oxidase I*, and *Larval Cuticle Protein*) were previously found to be differentially expressed in worker- versus queen-destined honey bee larvae (Corona et al., 1999; Evans and Wheeler, 1999), but the expression patterns differed from bumble bees. Larger scale studies using genome-wide gene expression will be necessary to comprehensively examine the processes underlying caste differentiation and how conserved these are across Hymenoptera.

6.4 Physiological and social factors affecting caste determination

As noted above, there is a conflict between the queens and workers over the timing of gyne production (Bourke and Ratnieks, 1999). Theoretically, the queen, the workers, and the developing female larvae can play a role in determining the fate of the larvae. The queen may modulate caste determination by adjusting the type of egg she lays, e.g., changing oocyte composition both with respect to nutritional reserves and molecular factors that may affect gene expression. The queen may also indirectly affect larval development by regulating worker brood care behaviour (although this can be counteracted by worker behaviour if it negates their self-interest). The workers, being the prime caretakers of the brood, may affect larval destination by regulating its nutrition, in particular in species where there is a clear size difference between the castes. Overfeeding may lead to queen determination, while underfeeding may lead to worker determination. Workers can also potentially mix endogenous factors with the food which induce/inhibit particular developmental pathways. Theoretically, the best interest of the totipotent diploid larva is to develop into queen, since as queen it will be more related to its own female (would be queens) offspring

($r=0.5$) than to female offspring of any of its sister gynes $r<0.5$) (Bourke and Ratnieks, 1999). In pocket maker bumble bee species, larvae can compete over food and achieve differential growth rate so that some develop into large workers, i.e., queens. In pollen storing species, however, larvae are fed progressively and have little or no control over their growth rates. Nonetheless, they can potentially still eavesdrop on colony environment and direct their own fate accordingly. Below, we discuss some of the factors that may affect larval developmental pathway to either worker or queen.

6.5 Nutrition as a factor affecting caste determination

In most species, the queen is larger in size than workers, suggesting that feeding rates and feeding amounts may affect caste determination. Support for this hypothesis comes from several studies, mostly in species in which caste determination occur only in their last stages of larval development, e.g., the European *B. pratorum* (Free, 1955), *B. hypnorum* (Free, 1955; Roseler, 1970), and *B. pascuorum* (Reuter and Schwammberger, 1999) and the North American *B. rufocinctus* and *B. ternarius* (Plowright and Jay, 1968). Moreover, manipulation of larval feeding rate in *B. rufocinctus* demonstrated that well-fed larvae developed into queens, whereas underfed larvae developed into workers (Plowright and Jay, 1977). Likewise, in *B. hypnorum*, if worker-destined larvae are well fed, they develop into queens (Roseler, 1989).

In contrast, caste determination in *B. terrestris* occurs early in larval development, and so far no effect of diet was found. Feeding duration of female larvae increases with colony development, with workers of the third brood receiving longer duration feedings compared to the first and second brood. However, there were no differences in feeding duration between queen- and worker-destined larvae (Ribeiro, 1999). Feeding frequencies were rather variable across individuals over time but overall queen larvae in the last phase of development were fed more frequently than worker larvae (Ribeiro et al., 1999). This is probably the consequence of caste determination, since in *B. terrestris* caste determination occurs at earlier phase of development. One possible explanation is that workers respond to larval 'hunger signal' and feed larvae. Queen larvae, due to their larger size, may metabolize the food quicker and therefore signal for food more frequently (Pereboom et al., 2003).

Food type may constitute another factor that directs caste differentiation, as was shown for the honey bee (Kamakura, 2011). Analysis of the three major components in larval food, sucrose, pollen, and proteins, revealed that

although workers add proteins (presumably gland derived) to the regurgitated food, there were no differences in food composition between worker- and queen-destined larvae (Pereboom, 2000). These analyses however were too general to conclusively state that workers do not add some 'determination factors' into the larval food.

6.6 The effect of queen-worker conflict on caste determination

In most species of bumble bees, gyne production occurs towards the end of colony life cycle, and as seen in *B. terrestris* is tightly linked with the eruption of queen-worker conflict over male production (Cnaani et al., 2000a; Duchateau and Velthuis, 1988). As discussed above, queens and workers have different optima regarding the timing of gyne production, providing the basis for another social conflict, and raising the question of who controls the onset of gyne production.

Support for the 'social effect on caste determination' came from experiments where egg cells from very young colonies (thus normally destined to develop into workers) were transplanted into host colonies at various stages of development. Eggs transplanted into host colonies that were up to 5 days before the onset of the CP developed into workers, whereas those transplanted into colonies closer to the onset of the CP developed into queens (Cnaani et al., 2000a). Evidence for queen control over gyne production came from experiments where uncommitted larvae were reared with queens either before or after the CP in combination with either naïve or experienced (in rearing gynes) workers. Irrespective of worker type, larvae in the presence of a pre-CP queen mostly developed into workers compared to larvae in the presence of CP queens that developed mostly into queens (Cnaani et al., 2000a). Interestingly, in this experiment, experienced workers were better at rearing queens in the presence of CP queens. Thus, it is tempting to speculate that the queen effect is nevertheless mediated through worker behaviour and that experienced workers may supplement the larvae with some nutritional factors that are absent in naïve workers.

Earlier studies of caste determination in *B. terrestris* hypothesized that the queen controls the developmental fate of the larvae through a pheromone, and that as the colony grows, the effect of this pheromone declines and the developing larvae consequently switch to queen development (Roseler, 1970, 1976, 1989). Supporting the idea of queen influence on larval fate is the fact that uncommitted larvae reared under QL conditions, thence in the absence of the putative queen pheromone, develop mostly into

queens (Cnaani et al., 1997; Roseler, 1970). Uncommitted larvae that were reared with workers but isolated from the queen developed into gynes, whereas if they were separated from the queen by a queen excluder (allowing workers to travel between compartments), they developed into workers. However, if some of the workers were transferred every 24 h between the QR and QL compartments (completely separated to prevent any contact between compartments), the larvae in the QL compartment still developed into queens. The author concluded that the queen produces a pheromone that affects worker behaviour directing them to rear either queens or workers, and that this pheromone is not transferable to the workers that were not in direct contact with the queen (Roseler, 1970). This study further revealed that queens taken from colonies at the CP were not able to inhibit gyne production, suggesting a decline in pheromone capacity. The correlation between the onset of gyne production and the onset of the CP leads to a further conclusion; the same pheromone inhibits both queen determination and worker reproduction, and that the queen stops producing the pheromone as a consequence of her loss of dominance (Duchateau and Velthuis, 1988; Roseler, 1989). This conclusion was later challenged by Bourke and Ratnieks (2001) on theoretical grounds.

Queen versus worker effects on caste determination were further investigated by creating young colonies headed by old queens and, vice versa, old colonies headed by young queens (Alaux et al., 2005). When colony composition was completely standardized (i.e. each colony composed of only 20 callow workers), queen age was a decisive factor in caste determination. Larvae housed with a young queen developed into workers, while larvae housed with an old queen developed into gynes. When the host colony social composition remained unchanged (i.e. the number of workers and their age and the larvae/worker ratios), the results were mixed. In young colonies that were headed by old queens, gyne production was advanced, but the inverse was not true. Old colonies headed by a young queen still produced queens. It is possible in this case that workers from old colonies underwent an irreversible change that rendered them insensitive to the change in the queen's pheromonal output, or that the unusually high number of workers signalled to the queen that the colony is at an advanced stage of development and induced her to change her pheromone output. This latter explanation is consistent with earlier findings that doubling the worker population advanced the onset of gyne production (Bloch, 1999). It was further revealed in this study that the queen calculates her age from the first worker emergence, that is, from the onset of the eusocial phase (Alaux et al., 2005).

6.7 Is there a queen pheromone that regulates caste determination?

The evidence presented above suggests that the regulation of caste determination in *B. terrestris* is pheromonally mediated, but neither the nature nor the glandular source of this pheromone is known. Below we list, on the basis of the present knowledge, the characteristics of such a putative pheromone(s) and suggest its possible nature and glandular source.

The mesh and queen excluder experiments indicate that the pheromone is not volatile and is not transferable between workers (Roseler, 1970), suggesting that the queen, while travelling in the nest, may add some of the secretion to the developing larvae or at least deposit the pheromone in the cell wax. The queen exchange experiments further indicate that the queen's pheromonal output changes both with age and social conditions. Young queens are able to direct larval development into workers, and if we postulate that queen development is the default developmental pathway (Cnaani and Hefetz, 2001) this is presumably achieved via inhibition of queen development. Since in the presence of old queens this presumed inhibition is alleviated, it can be concluded that such queens either stop producing the pheromone or change its composition. Recent analysis of labial gland secretion of queens and workers under various social conditions points to its possible role in regulating caste determination. The secretion is endowed with hydrocarbons and a plethora of esters of fatty acids (Amsalem et al., 2014a). Of particular interest is a series of dodecyl esters ranging from dodecyl hexanoate to dodecyl octadecenoate, which exhibit complex patterns. In queens, they are in high amounts in virgin and in young pre-CP fertile queens, but decline considerably (by 80%) in queens at the CP. In parallel, there is a change in the corresponding acids, which are high in old CP queens and low in young pre-CP queens. These changes are uncorrelated with ovarian activation since ovarian activation was low in virgin queens and comparably high in the two queen types (pre-CP and CP). This suggests that as queens' age and/or the changes in colony social status, the esters are hydrolyzed and consequently the quantities of the corresponding acids increase. It is tempting to postulate that these changes are perceived by either the workers or the larvae or both, thus influencing, directly or indirectly, the developmental fate of the larvae.

Unlike in queens, the changes in the labial gland dodecyl esters of workers are correlated with ovary activation. Sterile workers possess relatively high amounts of dodecyl esters (but still much lower than in queens), irrespective of whether taken from pre-CP or CP colonies, but they decline

by more than half in egg-laying workers. We do not fully understand the meaning of these differences, but it may point to a dual function of these esters and the possibility that they work in concert with other pheromones (e.g. Dufour's gland secretion).

Whether the decline in dodecyl esters and corresponding increase in acids precedes the CP and queen production, or not, requires a more precise time-dependent study. Nonetheless, the findings that workers that are exposed to either queen larvae or queens taken from gyne-producing colonies advance the competition point (Alaux *et al.*, 2006), coupled with the findings that the larvae switch to the queen developmental pathway a few days before the onset of the CP indicate that the switch in queen pheromonal composition precedes and triggers all of these events. The question of whether the queen exerts her regulation directly on the larval development or through affecting worker brood-care behaviour, as well as the question of whether the same pheromone is responsible for regulating worker reproduction and gyne commitment, remain open.



7. CONCLUSIONS AND FUTURE DIRECTIONS

In addition to their importance in pollination and agriculture, bumble bees are also an excellent system to examine and test the proximate and ultimate mechanisms underlying the organization and evolution of social behaviour. While sophisticated theories have been developed regarding the ultimate mechanisms driving social behaviour during the different colony phases, our understanding of the associated communication, physiological, and genomic mechanisms remain largely in their infancy. However, detailed knowledge of these mechanisms can greatly inform our understanding of the evolutionary processes; for example, until empirical studies revealed the presence of a 'sterility signal' in bumble bee workers, such a communication signal was not predicted. Below, we highlight outstanding questions in the field of bumble bee biology.

- (1) What are the factors and mechanisms underlying the myriad of life history strategies used by different bumble bee species? How do ecological, social, and physiological factors interact to shape these different strategies, and to what extent are these life history strategies evolutionarily constrained or plastic?
- (2) What is the nature of the queen fertility signal? Despite years of research in this area, we are still lacking a complete understanding of the nature

- of the social signal underlying queen reproductive dominance during the pre-CP phase, and how this dominance is lost during the CP phase.
- (3) How did the worker sterility signal evolve? Is this signal present in other species that display reproductive dominance hierarchies among workers or queens?
 - (4) How do social cues interact with physiological and genetic mechanisms to establish and maintain dominance hierarchies, ovarian activation, and egg laying?
 - (5) How have ancient pathways (genetic toolkits) been modified and reorganized during the evolution of social behaviour? For example, *vg* has evolved novel functions in highly eusocial honey bees compared to solitary species, and the regulation of *vg* by JH has changed concurrently. In bumble bees, the function of JH and *vg* and their regulatory interactions appear to represent an intermediate state (Amsalem et al., 2014b). How have other pathways been modified to generate complex social behaviours?
 - (6) Are there epigenetic mechanisms that underpin social behaviour? Recent studies suggest that DNA methylation regulates worker reproduction (Amarasinghe et al., 2014), but the underlying mechanisms (which genes? How does methylation impact gene function?) remain to be determined. Is there intra-genomic conflict between maternally derived and paternally derived alleles (Queller, 2003) and if so, how is this regulated?
 - (7) What social factors trigger caste differentiation? What is the signal produced by the queen that alters larval developmental fate? Is there also a signal produced by the queen or larvae that alters worker feeding-behaviour? What molecular mechanisms trigger caste differentiation? Are these conserved across Hymenopteran species? Are these derived from diapause-related pathways (Hunt et al., 2007)?

Addressing these questions will require an integrative multi-disciplinary approach, spanning behaviour, neurobiology, physiology, chemical ecology, and genomics. Recent advances in genomic resources (Sadd et al., 2015), genomic technologies (including high-throughput sequencing of transcriptomes, genomes, and methylomes), and genetic technologies allowing the modulation of gene function (such as RNAi, TALENs, and CRISPRs) have greatly facilitated our ability to probe the molecular mechanisms underlying these behaviours and examine how these have evolved across species. Finally, we should take advantage of the breadth and diversity of natural histories and life history strategies displayed by different bumble

bee species, to better understand the mechanisms underlying both social behaviour and adaptation to different ecological niches.

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