Colony Life History in the Bumble Bee *Bombus terrestris*: Interactions, Timing and Control.

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Thesis submitted for the degree of Doctor of Philosophy

University of East Anglia

School of Biological Sciences

December 2013

Thesis abstract

The evolutionary success of organisms is dependent on adaptive life histories, but the mechanistic control of life history traits is not often studied from an evolutionary perspective. One fascinating area with the potential to advance the understanding of life history regulation in an evolutionary context is the eusocial insects, since their colonies can themselves be regarded as possessing life histories. This is because whole colonies must develop and reproduce effectively in order to pass on the genes of their members. However, as not all colony members have the same fitness optima for colony life history traits, conflict can exist over the control of these traits. Furthermore, colony traits may respond differently to the external environment than in individual organisms, because affecting the life history of colony members might not have corresponding effects on the life history of whole colonies. In this thesis, I use laboratory experiments with the bumble bee *Bombus terrestris* to investigate the control of eusocial insect colony life history, with a focus on the interactions that bring about control over timing. Specifically, I reveal queen control over the onset of male production; show that colonies do not differ over colony development in their response to natal or non-natal worker laid eggs; demonstrate that higher temperature increases the productivity, but not longevity, of individuals and colonies; and find that foraging gene expression in queens may be linked to colony establishment. Taken together, these findings advance the understanding of life history and social evolution by illuminating processes at behavioural and molecular levels which regulate colony life history in eusocial insects. Furthermore, I discuss how this research has potential applications for the ecological and commercial management of bumble bees, which are key pollinators of crops and wild flowers.

Chapter contributions and publication

All parts of this thesis have been written by Jacob Holland, in consultation with Andrew Bourke (and with David Collins for the methods of Chapter 5). Estimates of approximate percentage contributions to the initial concepts, planning, conducting (data collection) and analyses of the experiments included in each thesis data chapter (2-5) are given below. The publication status of the findings presented in each data chapter is also given.

Chapter 2: published, in largely the same format, as: Holland JG, Guidat FS, Bourke AFG (2013) Queen control of a key life-history event in a eusocial insect. *Biology Letters 9:20130056*

- Concept: **Jacob Holland 50%**; Andrew Bourke 50%
- Planning: **Jacob Holland 70%**; Andrew Bourke 30%
- Conducting: **Jacob Holland 85%**; Florian Guidat 15% (genotyping service provided by the John Innes Centre, Norwich Research Park, Norwich, UK)
- Analysis: **Jacob Holland 90%**; Andrew Bourke 10%

Chapter 3: not yet published

- Concept: Lorenzo Zanette 50%; Andrew Bourke 50%
- Planning: Lorenzo Zanette 30%; Andrew Bourke 30%; **Jacob Holland 20%**; Tulio Nunes 20%
- Conducting: **Jacob Holland 50%**; Tulio Nunes 50%
- Analysis: **Jacob Holland 90%**; Tulio Nunes 10%

Chapter 4: not yet published

- Concept: **Jacob Holland 60%**; Andrew Bourke 40%
- Planning: **Jacob Holland 70%**; Andrew Bourke 30%
- Conducting: **Jacob Holland 70%**; Cameron Stables 15%; Rachel Turner 10%; Peter Higgins 5%.
- Analysis: **Jacob Holland 100%**

Chapter 5: not yet published

- Concept: **Jacob Holland 40%**; Andrew Bourke 40%; David Collins 20%
- Planning: **Jacob Holland 40%**; David Collins 40%; Andrew Bourke 20%
- Conducting: **Jacob Holland 40%**; David Collins 40%; Faaziah Chishty 20% (qPCR service provided by qStandard, UCL, London, UK)
- Analysis: **Jacob Holland 90%**; David Collins 10%

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Acknowledgements

First and foremost, I wish to thank my supervisor Andrew Bourke, whose mental endnote library usually proved far more extensive and useful than the one stored on my computer. His unfailing ability to illuminate complex science with clarity and simplicity always proved invaluable, and his words of encouragement were always well timed. I also wish to thank all members and visitors of the Bourke group, past and present, with whom I had the great pleasure of sharing science and friendship (and from whom I received a great deal of help), including most especially: Edward Almond, Faaizah Chishty, David Collins, Christiana Faria, Lucy Friend, Florian Guidat, Tim Huggins, Peter Higgins, Tulio Nunes, Cameron Stables, Rachel Turner and Lorenzo Zanette. Thanks also go to my secondary supervisor Matt Gage, who provided a useful outside perspective in discussions throughout my PhD, and to my thesis examiners Jenny Gill and Mark Brown, whose comments improved the thesis.

I also have immense gratitude for all those making up the UEA Centre for Ecology, Evolution and Conservation. Together, they fostered an incredibly supportive and companionable environment, which meant a great deal at those times when it felt like the workplace was my main residence. This was particularly true of my office mates in the 'Bambi lab' and 'Stag lab', most notably: Conrad Gillet, Christiana Faria, Henry Fergusson-Gow, Becky Laidlaw and Karl Phillips. Karl, in fact, deserves special mention, because we saw through the joys and stresses of our PhDs almost in tandem; a great source of comfort and encouragement.

I thank the Natural Environment Research Council for funding my research and, together with the UEA, helping me to pursue knowledge of the natural world. I further thank the International Union for the Study of Social Insects, Pamela Salter and the Company of Biologists for funding my attendance at several conferences, allowing me to connect with scientists from around the world and work my way into the (sociable) social insect community. Thanks also go to all those (too numerous to name) who provided copies of papers, unpublished data, new ideas and technical help.

I'm ever thankful for the support of all of my family, especially Mum and Dad, who stuck by me even when what I was doing seemed a little out of the ordinary. Finally, I am humbly grateful for my wife Malissa, through whose unwavering love and devotion I have been kept sane.

Chapter 1

Life history in eusocial insects and bumble bees

Life history in eusocial insects

Life history: proximate and ultimate perspectives

A common factor throughout the evolution of living things is the selection to develop and reproduce in life cycles that maximise an individual's contribution to future generations. The forms taken of these life cycles vary immensely across organisms, but can be understood and compared by considering a series of key 'life history traits', including: size at birth; growth pattern; age at maturity; size at maturity; number, size and sex ratio of offspring; age- and size-specific reproductive investments; age- and size-specific mortality schedules and length of life (Stearns 1992; Roff 2002). Approaches for considering these traits involve both 'ultimate' and 'proximate' perspectives, as outlined by Tinbergen (1963). These perspectives distinguish reasoning about where a trait originated and why it has been selected for in evolutionary terms (ultimate), from reasoning about how the trait develops and works mechanistically within the lifetime of an individual (proximate). Although the two perspectives are obviously linked, it often makes sense to consider them separately (Alcock and Sherman 1994). On a proximate level, life history traits are controlled both by effects of genotypic variation, and effects of variation in the external environment (phenotypic plasticity) in the way organisms develop and behave (Nylin and Gotthard 1998; DeWitt and Scheiner 2003). Since different conditions will generate differential optimal life history traits at both an evolutionary and an organismal timescale, these two pathways together allow organisms the potential to generate adaptive life histories (Williams 1966b; Stearns 1976; DeWitt and Scheiner 2003). A key series of questions therefore arises concerning how life history is controlled by proximate mechanisms in different organisms. Since ultimate thinking can predict mechanisms, and understanding mechanisms can inform ultimate thinking, the details of these proximate mechanisms in different types of organism are of great importance to evolutionary biology (Sinervo and Svensson 1998; DeWitt and Scheiner 2003). However, these mechanisms have rarely been considered from an evolutionary perspective (Flatt et al. 2005).

Eusociality and colony life history

Living things exist at different levels of social organisation; most notably, unicellular organisms, multicellular organisms, and eusocial societies (colonies of individuals with a reproductive division of labour, cohabitation of overlapping generations, and cooperative brood care; Wilson 1971) (Maynard Smith and Szathmáry 1997; Bourke 2011). The first two of these levels are considered individual organisms (e.g. a bacterium or an animal), and exhibit life history traits of their own. Here, I follow previous authors in also considering eusocial societies as organisms (or superorganisms) (e.g. Wilson 1985; Maynard Smith and Szathmáry 1997; Queller and Strassmann 2009; Bourke 2011), because the synergy and division of labour between individuals is analogous to the synergy and division of labour between cells in a multicellular organism. Treating whole colonies as organisms with regard to life-history can be thought of as more useful than trying to define the life-histories of individuals within colonies, many of which may not themselves reproduce. Because eusociality is a condition found in many species across the animal kingdom (Wilson 2000; Bourke 2011), understanding the proximate regulation of colony life history in eusocial systems will lead to unique developments in evolutionary biology. Furthermore, it is likely to yield insight into other levels of social organisation, such as multicellular organisms, if analogous mechanisms are used.

Eusociality is most marked in the Hymenoptera (ant, bees and wasps) and termites, which in the case of some ant and termite species include colonies consisting of over one million individuals, with complex interactions and interdependence (Wilson 2000; Bourke 2011). For this reason, eusocial insects are the most obvious candidate for understanding the control of colony life history. Despite this, Bourke and Franks (1995) suggested that the understanding of life history in eusocial insects was at a particularly early stage. Although this comment was made almost two decades ago, many questions still remain as to how the unique life history patterns exhibited by eusocial insect colonies evolved and are proximately regulated (e.g. Remolina and Hughes 2008; Lopez-Vaamonde et al. 2009; Poitrineau et al. 2009). The main focus of this thesis will be to investigate the life history of eusocial colonies, with particular emphasis on the interactions within the colony that give rise to control over the timing of colony life history. By considering these factors together, I will advance understanding of the mechanisms underlying colony life history in eusocial insects and give insight into how it may have evolved.

In order to understand life history at the colony level, it is important to understand the individuals that make up colonies. Eusocial insect colonies mostly consist of one or more reproductive individuals, often called ‘queens’ (and also ‘kings’ in the case of termites), and a number of non-reproductive or rarely-reproductive individuals, usually called workers, which help to rear the offspring of queens. Workers themselves are, in many species, generally the offspring of the colony queen(s), and it is this relatedness between individuals which is thought to have been crucial for the evolution of eusociality and its subsequent maintenance (Hamilton 1964; Hughes et al. 2008; Boomsma 2009; Bourke 2011). In the eusocial Hymenoptera, workers are females which are generally unable to mate and do not become queens, although in some cases workers can reproduce by parthenogenesis (asexual reproduction). Worker parthenogensis includes ‘arrhenotoky’, which produces haploid male offspring (Heimpel and de Boer 2008), and the far rarer ‘thelytoky’, which produces diploid female offspring (Rabeling and Kronauer 2013). A combination of male haploidy and female diploidy (termed ‘haplodiploidy’) is the normal condition in the eusocial Hymenoptera, and queen-produced males are produced by arrhenotoky, whereas workers are produced by sexual reproduction (except in the rare cases of reproduction by thelytoky) (Heimpel and de Boer 2008). Except in some facultatively eusocial species (i.e. where individuals may or may not live in eusocial colonies during their lives), workers constitute the main workforce of the colony and perform tasks, such as brood rearing and foraging, regarding which there is a division of labour (i.e. individuals specialise in different tasks within the colony) (Wilson 1985; Bourke and Franks 1995; Anderson and McShea 2001; Johnson 2010). It is these individuals that must interact to create an organised colony-level development that allows successful growth and reproduction (Oster and Wilson 1978; Wilson 1985; Bourke and Franks 1995; Wharton et al. 2007; Bourke 2011).

In general, the development of a eusocial insect colony can be separated into three stages: the foundation stage, the ergonomic growth stage, and the reproductive stage (Oster and Wilson 1978; Bourke and Franks 1995). In the foundation stage, a queen (or an association of several queens) establishes a nest and begins producing workers, helping to rear them by foraging for food, or by providing stored resources (Brown and Bonhoeffer 2003; Cronin et al. 2012). After workers have been produced, a division of labour occurs between the queen(s) and workers, whereby the workers become the main foragers and brood rearers, and the queen devotes much of her energy to egg-laying (Oster and Wilson 1978). This is called the ergonomic growth stage because it

allows the colony to grow by increasing the number of workers. Eventually, successful colonies will reach a reproductive stage, which is characterised by the production of individuals capable of sexual reproduction (sexuals).These sexuals will mate with individuals from other colonies in order to establish new colonies. In the eusocial Hymenoptera, these sexuals consist of new queens (termed ‘gynes’) and males.

In species with an annual colony cycle, the colony will decline after the reproductive stage (Oster and Wilson 1978; Bourke and Franks 1995). Contrastingly, in species with a perennial colony cycle, the colony will alternate between ergonomic growth and reproductive stages, with the colony producing further sexuals in each favourable season (Oster and Wilson 1978; Bourke and Franks 1995). Colonies may also exhibit an orphanage stage (Franks et al. 1990; Bourke and Franks 1995), in which workers reproduce after the death of the colony queen. This ontogeny (developmental pattern over time) is largely analogous to that of multicellular organisms, with interactions between individuals being behavioural, rather than molecular, in nature (Wilson 1985; Maynard Smith and Szathmáry 1997; Bourke 2011). For example, the switch from ergonomic growth to the reproductive stage is similar to the shift between initial development and sexual maturation in a multicellular organism, because somatic growth is followed by reproductive growth (Poitrineau et al. 2009; Holland et al. 2013). The repeated switching between these phases in perennial colonies can also be compared to the seasonal growth and regression of gonadal development found in various animals (see Koukkari and Sothern 2006).

In table 1.1, I propose the key life history traits of a eusocial insect colony by drawing analogies to Stearns’ (1992) tabulation of individual-level life history traits outlined at the beginning of the chapter. These traits act as a guideline for studying eusocial insect life history and will facilitate comparisons with multicellular organisms. In the next two subsections I will consider ultimate and proximate perspectives on colony life history in eusocial insects, respectively.

Table 1.1 –The measurable eusocial insect colony analogues to the life history traits proposed by Stearns (1992).

Life-history trait	**Suggested colony-level analogous trait(s) in eusocial insects**
Size at birth	*Number of queens/size of queen(s)* *Time and size of first brood*
Growth pattern	*Time at which workers take over foraging and brood care* *Timing and rate of worker production*
Age at maturity	*Time at which reproductive stage begins*
Size at maturity	*Colony size at which reproductive stage begins*
Number, size and sex ratio of offspring	*Number and size of colonies descended from colony-produced sexuals*
Age- and size-specific reproductive investments	*Number, size and sex ratio of sexuals produced according to colony age and size*
Length of life (longevity)	*Time from foundation to death of the colony (taken either as death of last worker or death of queen –as workers can sometimes reproduce after the death of the queen, the death of the last worker may be a useful measure)*

Ultimate optima in colony life history

Despite cooperation, eusocial insect colonies also exhibit conflict between individuals (e.g. Hamilton 1964; Trivers and Hare 1976; Bourke and Chan 1999; Bourke and Ratnieks 1999; Bourke and Ratnieks 2001; Wenseleers and Ratnieks 2004; Ratnieks et al. 2006b; Helantera and Ratnieks 2009). Such conflict may arise when the inclusive fitness costs and benefits of alternative life-history strategies vary between different parties (e.g. queens and workers) within a colony (Trivers and Hare 1976; Franks et al.

1990; Ratnieks and Reeve 1992; Bourke 2011). This is because, put simply, individuals should be selected to exhibit traits which increase the propagation of their own genes. The reasoning here follows from the 'gene's eye view' approach that stipulates that only the fitness cost:benefit ratios of the lowest levels of selection (i.e. genes) should explain adaptations, because a gene that increases its own propagation will succeed (within limits) even if it does so at the expense of a higher level of selection (e.g. is detrimental to the individual or colony), as made explicit by Williams (1966a) and Dawkins (1976; 1982). Therefore, the behaviour or development of an individual in a colony which affects a colony life history trait should be selected for only if it results from genes which increase their own propagation. Phrased from an individual perspective, this means that a behaviour (in an actor) which affects the fitness of another individual (e.g. another nestmate; the recipient) should be selected for if the fitness benefit to the recipient multiplied by their relatedness to the actor is greater than the fitness cost to the actor (Hamilton 1964). Here, relatedness (*r*) refers to probability that the two focal individuals will share any given allele (Bourke and Franks 1995) and fitness benefits and costs refer to the gain or loss in the expected number of successful offspring produced (Dawkins 1982). Selection for a trait which benefits a related recipient at a cost to the actor (i.e. altruism) is termed 'kin selection', and the total fitness of an actor, including extra fitness it gains (or loses) through recipients as a result of actions performed, is termed 'inclusive fitness'. For individuals with a relatedness of 1 (such as cells in a multicellular organism), their genetic interests will always be aligned, but for individuals with a relatedness of less than 1, this may not always be true (Hamilton 1964; Ratnieks et al. 2006a; Bourke 2011). Whilst it is important to remember that the gene is the fundamental unit of selection, it is relevant to note that the cost:benefit ratios of lower levels of selection may well be in line with those of a higher level. For example, most genes in a multicellular organism will cooperate, and thus a gene's eye view is often similar to an organism's eye view, with some exceptions (Dawkins 1982; Bourke 2011). However, as stated above, the interests of different individuals in eusocial colonies may not always be so aligned and so an individual perspective (instead of a colony-level perspective) must be taken to avoid error in predicting ultimate benefits. For this reason, understanding the ultimate benefits of different life history strategies in eusocial insect colonies is complex and potentially more challenging than in multicellular organisms, because it must take into account the interests of different parties (kin groups) within the colony (e.g. queens and workers) (Franks et al. 1990). Below, several key life history traits in eusocial insect colonies are considered from this perspective.

Time at which workers take over foraging and brood care

The queens of many eusocial insects, including some eusocial wasps (Ross and Matthews 1991), some eusocial bees (Michener 2000) and some ants (Brown and Bonhoeffer 2003), forage at the foundation of a colony. In these species it is in the interest of the queen(s) to cease foraging as soon as workers are able to replace her, because this will allow the avoidance of risks associated with venturing outside the colony (Oster and Wilson 1978; Brown and Bonhoeffer 2003; Goulson 2010; Cronin et al. 2012). This should also generally be in the interests of workers because, in most cases, queens provide the main opportunity to pass on their genes (through the production of sexuals), and so are a valuable resource (Goulson 2010). Similarly, brood care by workers should normally be favoured by all colony members, because this may allow queens to devote more time to egg production, thus maximising worker (and eventually, sexual) production through division of labour (Oster and Wilson 1978).

Time/size at which reproductive stage begins

Since production of sexuals is critical for colony success, the correct timing of this event is of key importance (Oster and Wilson 1978; Beekman et al. 1998a; Poitrineau et al. 2009; Holland et al. 2013). Optimality theory predicts that there should be an optimum time to produce sexuals based on the seasonal availability of resources and mates and on the potential time remaining for colony survival (Alaux et al. 2005). As a greater number of workers should be capable of increasing the number of sexuals produced (Oster and Wilson 1978; Poitrineau et al. 2009), there should also be an optimal colony size at which to begin producing sexuals. In annual species, this strategy has been called the 'bang-bang' strategy, because of the maximisation of worker production first and then reproductives later (Oster and Wilson 1978). Although originally considered in colony-level terms, this bang-bang strategy can also be consistent with an individual level approach, as the diminishing returns of worker production means that there will come a point where further worker production is not beneficial to any within-colony party because of their minimal contribution to workload (Bourke and Ratnieks 1999). The overall optimal timing for sexual production should presumably not vary between queens and workers, but the timings of the production of specific sexes may be differentially preferred (see below). The fitness benefit of female larvae (which are totipotent) developing into new queens rather than workers may differ between queens, workers and larvae themselves, with larvae more often preferring to develop into new queens (Bourke and Ratnieks 1999; Wenseleers et al. 2003;

Ratnieks et al. 2006a; Hughes and Boomsma 2008). However, depending on the kin structure of the colony, it may still be beneficial to larvae to develop as workers instead (Bourke and Ratnieks 1999; Wenseleers and Ratnieks 2004).

Number, size and sex ratio of sexuals produced

Since sexuals are generally the only route for genes in individuals to spread to the next generation, all individuals should favour their production, and so there is unlikely to be a difference between the optimal number and size of sexuals produced between queens and workers. However, there may be differences in optima for the sex ratio and origin of the sexuals produced. For example, in the eusocial Hymenoptera, queens are equally related to their own sons and daughters, and so should prefer a 1:1 sex investment ratio in sexuals (Fisher 1930), but if all workers in the colony are descended from a singly mated single queen, they are more related to sisters (r = 0.75) than they are to brothers (r = 0.25) (or even sons and nephews) and so they should prefer a more female biased sex ratio (Trivers and Hare 1976; Ratnieks et al. 2006a). Furthermore, such workers will be more related to (worker-produced) sons (r = 0.5) or nephews (r = 0.375) than to (queen-produced) brothers (r = 0.25) (Ratnieks et al. 2006a). Therefore workers should in theory prefer the colony's resources to be spent on rearing a worker-produced male above rearing a queen-produced male on a one-to-one basis (Hamilton 1964; Bourke 1988), although this prediction assumes workers have information about when the queen is producing males (Beekman and Ratnieks 2003). Alternatively, if workers are descended from multiple queens or a multiply mated single queen, these sex ratio and male parentage optima may alter because workers will be less related to sisters and nephews and potentially less related to brothers (Wenseleers and Ratnieks 2006a).

Time from foundation to death of the colony

There are two possible uses of the term 'colony death' in eusocial insects: the death of the colony queen and the death of last worker. In this thesis, I will refer to these two events as queen death and colony death respectively, as the colony can still function and potentially rear sexuals after the death of the colony queen. The optimal timing of queen death may vary among colony members because the queen may prefer a later death than that preferred by workers (Bourke 1994; Bourke 2007) or potential replacement queens. In perennial colonies, colony death should be selected to occur only when the colony is unable to produce more sexuals, although it may also be beneficial to colony members if the colony would otherwise compete with successful

daughter colonies with a high reproductive output, since competition with daughter colonies can decrease fitness (Bourke and Franks 1995; West et al. 2002). In annual colonies, the timing of colony death after its single reproductive phase may be less important for colony members if the colony can no longer produce a large number of sexuals, and so colony longevity could be reduced by selection on genes that benefit the colony members early in colony development to the detriment of benefits after sexual production (an example of antagonistic pleiotropy) (Bourke 2007).

Proximate mechanisms in colony life history

Understanding the mechanisms regulating colony life history in eusocial insects is of great significance to evolutionary biology because it will help us to understand how eusociality evolved and is maintained. From a more applied perspective, understanding the colony life history of eusocial insects is of global practical importance because eusocial insects include both essential pollinators (e.g. O'Toole 1993; Chapman and Bourke 2001) and widespread invasive species (e.g. McGlynn 1999). In general, predicting the responses of organisms to the environment is likely to require an understanding of the individual-level processes that regulate such responses (Knight 2001; Norris 2004; Berger-Tal 2011). Thus, a better mechanistic understanding of how eusocial insect colonies are able to reproduce from one generation to the next is critical for the ecological and commercial management of these species. Despite this, few studies have shown how key colony life history traits are regulated mechanistically (Bourke and Franks 1995; Wharton et al. 2007). Fitness optima (see previous subsection) for different parties within colonies may not always be realised because the information required by colony members to make self-interested decisions and/or the power to control (affect by some behavioural or physiological mechanism) reproductive traits may not always be available to all individuals in the colony (Ratnieks and Reeve 1992; Bourke and Ratnieks 2001; Beekman and Ratnieks 2003; Ratnieks et al. 2006a; Helantera and Ratnieks 2009). Brief summaries of what is known about the regulatory mechanisms and control of key traits are given below.

Time at which workers take over foraging and brood care

Although queen foraging usually ceases a short time after worker emergence in many eusocial insects with foraging queens (Oster and Wilson 1978), the cues for this event or the physiological mechanisms driving it are not well understood (Brown and Bonhoeffer 2003). However, in *Pogonomyrmex* harvester ants, there is an association

between queen foraging and their ovarian activity, such that foraging queens have more active ovaries (Dolezal et al. 2013). The presence of brood themselves may also act as a cue since, in the bumble bee *Bombus terrestris*, circadian rhythmicity associated with foraging behaviour in queens is negatively associated with brood presence (Eban-Rothschild et al. 2011). However, whether these associative factors are causal in their influence on the cessation of queen foraging is not known. A possibly separate transition occurs between the queen providing brood care, and it being provided by the workers; in *B. terrestris* this appears to be regulated by worker presence and also produces an increase in queen reproduction (Woodard et al. 2013).

Time/size at which reproductive stage begins

In the eusocial Hymenoptera, the proximate factors causing female larvae to develop as gynes include nutrition (Smith et al. 2008; Kamakura 2011), and temperature (Bourke and Franks 1995). Several species of eusocial gall-forming aphids produce winged dispersers when the gall reaches maturity, and these forms can establish new colonies. The proximate cue for the production of these individuals may be the greater nutrition provided by the gall in its later stages of development (Keigo Uematsu, personal communication). In several species of the eusocial Hymenoptera and in at least one termite, the rearing of sexual larvae is repressed by queen pheromones (Vargo and Fletcher 1986; Alaux et al. 2005; Boulay et al. 2009; Matsuura et al. 2010), although this may take the form of a signal to larvae rather than a physiological restriction (Keller and Nonacs 1993; Bourke and Ratnieks 1999; Matsuura et al. 2010). However, in many of these cases it is not clear which parties in the colony (if any) control the shift to the sexual (or reproductive) production. In particular, almost nothing is known about the proximate causes of onset of colony-level haploid egg production in eusocial Hymenoptera, which is a prerequisite for the production of males in these systems.

Number, size and sex ratio of sexuals produced

The mechanisms associated with generating sex ratio have been fairly well studied in social insects. In general, queens are largely thought capable of regulating the number of eggs they lay of each sex (the ‘primary sex ratio’) and workers can control the numbers of developing individuals of each sex (secondary sex ratio) by preferential feeding (Trivers and Hare 1976; Bulmer and Taylor 1981; Beekman and Ratnieks 2003; Mehdiabadi et al. 2003; Aron 2012). Despite the wealth of research conducted in this area, little is yet known about how these mechanisms interact with control of the

timing of sexual production. One possible influence on sex ratio, worker reproduction, may be inhibited by the queen (e.g. Hoover et al. 2003; Alaux et al. 2004; Grangier et al. 2013), although, as with the development of larvae as queens (see previous subsection), this may be the result of a signal rather than direct physiological inhibition (Keller and Nonacs 1993; Heinze and d'Ettorre 2009; Kocher et al. 2009; Kocher and Grozinger 2011; Holman et al. 2013). Also, worker reproduction may be policed by the queen or other workers via egg-eating (Wenseleers and Ratnieks 2006a; van Zweden et al. 2009a) or aggression towards laying workers (Bloch and Hefetz 1999; Teseo et al. 2013).

Time from foundation to death of the colony

Colony queens in some species may be killed by workers (Bourke 1994). Although factors affecting the longevity of social insect queens and workers have been studied in a number of species (Calabi and Porter 1989; Corona et al. 2005; Remolina and Hughes 2008; Hoover et al. 2012), little is known about factors affecting the longevity of the colony as a whole.

Open topics in colony life history

A number of key questions relating to the regulation of colony life history in eusocial insects remain to be answered. In particular, it will be important to develop and add to the current knowledge of within-colony interactions given above, and consider how these behaviours proximately control life history traits at a colony level. For example, very little is known about the control of the timing of colony growth, colony sexual maturity and colony longevity. Underpinning the behavioural interactions that regulate colony life history are the physiological and molecular mechanisms that occur within individuals. Thus a full understanding of colony life history will also require knowledge of these internal mechanisms, with the eventual aim being a fuller integration of the molecular, physiological, organismal and social levels of biological explanation.

In exploring mechanisms in biological systems, model organisms allow an integrated perspective because new data can be interpreted within the context of previously known biological information about the organism in question. For this reason, the establishment of model systems is a crucial building block in mechanistic biological research. In the next section, I argue that a well-suited model system for studying colony life history traits in eusocial insects, particularly in annual species, is the bumble bee *Bombus terrestris*.

Life History in bumble bees

Bumble bees (*Bombus* spp.) represent an ideal system for studying key life history traits of eusocial insect colonies for a number of reasons. Firstly, most species possess an annual colony cycle (Alford 1975; Goulson 2010) and are also readily reared in the laboratory (Velthuis 2002). This means that many traits can be studied and manipulated in controlled conditions over the entirety of the life cycle, both in a manageable time-frame and with relative ease. Secondly, bumble bees, and in particular *Bombus terrestris*, are well studied in the laboratory and a number of clear life history events have been identified (outlined below) (Duchateau and Velthuis 1988; Duchateau et al. 2004; Goulson 2010). Thirdly, detailed research has also yielded tools for the study of *Bombus terrestris* on a molecular level (Estoup et al. 1995; Estoup et al. 1996; Pereboom et al. 2005; Colgan et al. 2011), and a genome is near to publication (Sadd et al. unpublished data). Fourthly, there is a good understanding of the ecology of many bumble bee species (e.g. Alford 1975; Goulson 2010; Prŷs-Jones and Corbet 2011), allowing life history data to be evaluated in an ecological context. Fifthly, bumble bees are key global pollinators of both commercial crops and a large number of wild flowers (Goulson 2010). The importation of bumble bees (particularly *Bombus terrestris*) for agricultural pollination (Velthuis 2002) has also rendered them invasive pests (Donovan and Wier 1978; Hingston and McQuillan 1999; Matsumura et al. 2004; Dafni et al. 2010) (Donovan and Weir 1978; Hingston and McQuillon 1999; Matsumura et al. 2004; Dafni et al. 2010), and so bumble bees are highly important both ecologically and economically in their own right.

In terms of social evolution, bumble bees are sometimes classed as primitively eusocial (Bourke 1999; Johnson and Linksvayer 2010) (except in the case of the socially parasitic subgenus *Psithyrus*), because they have a colony size consisting of no more than a few hundred workers, queens which are morphologically similar to workers (although larger), and single queens which are generally singly mated (Goulson 2010). This separates them from other well studied social insects such as honey bees (*Apis* spp.), leaf cutting ants (*Atta* and *Acromyrmex* spp.), and the imported red fire ant (*Solenospis invicta*), all of which are generally considered advanced eusocial species because they possess large colonies, morphologically distinct queen and worker castes and multiply mated queens or multiple queens per nest (Bourke 1999; Johnson and Linksvayer 2010). Thus, bumble bees represent a system which may be regarded as en-route to increasing social complexity, and so offer an opportunity to explore the

evolution of regulation in eusocial insect colonies. The typical life history of *Bombus terrestris* (and probably most temperate eusocial bumble bees) is outlined below.

During mating in autumn, each queen will mate with only a single male, i.e. monandry (Schmid-Hempel and Schmid-Hempel 2000) (although in some other *Bombus* species, queens can mate with two or more males (Brown et al. 2003)). After mating occurs, males die and mated queens hibernate (diapause) before establishing a colony. Queens emerging from hibernacula first locate nest sites, in which they store nectar collected from foraging (as honey). Each new nest site is established by a single foundress queen and the colony will continue to its death with this foundress as the only mated queen ('monogyny'). The queen then produces diploid brood in a series of batches, the development of each batch overlapping temporally with the laying of the next (Heinrich 1979). As this brood develops into workers, the amount of time the queen must devote to rearing larvae decreases (due to rearing efforts by worker-nurses and foraging efforts by worker-foragers; the ergonomic growth stage). At some point, the colony switches to the reproductive stage in two key events: the 'switch point' (Duchateau and Velthuis 1988; Duchateau et al. 2004), and the 'onset of gyne development' (Cnaani et al. 1997; Cnaani et al. 2000; Alaux et al. 2005). The switch point is defined as the time at which the queen begins to lay haploid (male destined) eggs. At the switch point, the queen increases the proportion of haploid eggs until (potentially) only haploid eggs are laid (Van Der Blom 1986; Duchateau and Velthuis 1988; Lopez-Vaamonde et al. 2003; Duchateau et al. 2004; Lopez-Vaamonde et al. 2009). In some *B. terrestris* populations, there is an observed bimodality in the timing of the switch point (Duchateau and Velthuis 1988; Inoue et al. 2010). However, this is not true of all populations (Duchateau et al. 2004; Lopez-Vaamonde et al. 2009). The onset of gyne development is the time at which diploid larvae begin development as gynes instead of workers (Cnaani et al. 1997; Cnaani et al. 2000; Pereboom et al. 2003; Alaux et al. 2005), and generally occurs shortly after the switch point (Duchateau et al. 2004; Lopez-Vaamonde et al. 2009), although many colonies do not produce any gynes (Duchateau et al. 2004; Lopez-Vaamonde et al. 2009; Inoue et al. 2010).The competition point is another key colony life history event, which occurs usually after the switch point and the onset of gyne development (Van Doorn and Heringa 1986; Duchateau and Velthuis 1988; Bloch 1999; Duchateau et al. 2004; Lopez-Vaamonde et al. 2009). At this time, worker egg laying and sometimes aggression (both worker-queen and worker-worker) begins, and so it approximately corresponds to the orphanage stage (although the queen is usually still alive at the time of the CP). Since

the publication of Duchateau and Velthuis's paper (1988) describing these events, many authors have viewed these life history events as highly important parameters in the colony development of annual bumble bees (e.g. Duchateau and Velthuis 1989; Shykoff and Müller 1995; Bloch 1999; Beekman and Van Stratum 2000; Bourke and Ratnieks 2001; Lopez-Vaamonde et al. 2003; Duchateau et al. 2004; Alaux et al. 2005; Alaux et al. 2006; Gosterit and Gurel 2009; Lopez-Vaamonde et al. 2009; Amsalem and Hefetz 2011; Zanette et al. 2012). After the competition point, colonies eventually begin to decline until colony death occurs at the end of the season (in most populations of *B. terrestris*), which usually takes place in the autumn. Before colony death, queens may be killed by workers (Bourke 1994). No direct trade-off appears to exist between queen longevity and reproduction, since longer lived queens produce more sexuals (Lopez-Vaamonde et al. 2009). Mated gynes (queens) diapause for 6-9 months (Alford 1969) in temperate climates, before emerging to establish a colony in the spring.

In other *Bombus* species, the timing of colony life history varies; for example, the production of sexuals occurs earlier in colony development in *B. terricola* and *B. lucorum* (Müller et al. 1992), and longevity of colonies is greatly reduced in species such as *B. pratorum* and *B. hortorum* (Prys-Jones 2011). This is likely to result not only from differences in geographical range (e.g. Michener and Amir 1977; Buttermore 1997; Rasmont 2008), but also differences in traits such as colony size and worker longevity (Müller et al. 1992). Bumble bee species also vary in other traits which may affect life history, including the method of larval feeding and the degree of specialisation on food plants. In terms of larval feeding, *Bombus* species are divided into pollen storers and pocket makers. Pollen storers (such as *B. terrestris*) store pollen in wax cups in the nest and feed larvae individually, whereas pocket makers provide pockets of pollen under brood cells, allowing larvae to compete for resources. The competition between larvae in pocket makers is thought to lead to greater variability in colony size compared to pollen storing species (Sladen 1912; Prŷs-Jones and Corbet 2011). In terms of food specialisation, some species (such as *B. terrestris*) are short tongued and may forage on a large number of different plant species. By contrast, long tongued species tend to be specialists, meaning that their life history may be more tailored towards matching the timing of specific food plants (Goulson 2010).

Thesis aims

In order to better understand the proximate control of colony life history and critical events in eusocial insect colony cycles, a laboratory experimentation approach has been chosen to address a number of key questions. Although working with colonies in the laboratory may not accurately simulate conditions in nature, this can itself be an advantage. Laboratory experiments can provide controlled research which allows the focused understanding of basic relationships between life history traits and a small number of key variables. Without this background information, data from the field can be difficult to interpret, because field conditions often vary in multiple factors which cannot be controlled. Thus, lab and field studies work in complement, because the knowledge of basic relationships in the laboratory can be used to make prior estimates and generate hypotheses about what can be expected in the field. In addition, observations or inferred relationships between variables in the field can be tested more rigorously using laboratory experiments. Furthermore, laboratory experiments are necessary to investigate the role of factors which would be difficult or impossible to investigate in the field, such as levels of gene expression. Such studies are therefore necessary to bridge the gap in understanding between fine scale molecular processes and phenotypic effects at the ecological scale.

As outlined above, a number of colony life history traits offer the potential for study into the mechanisms of colony development. In this thesis, I have taken a broad approach by investigating a number of these traits, and using a variety of laboratory techniques to do so. As far as was possible, the data chapters (Chapters 2-5) have been arranged based on the chronological occurrences of the main life history events they respectively focus on, namely: the switch point (Chapter 2), the competition point (Chapter 3), colony death (Chapter 4) and colony foundation (Chapter 5). Although there are a multitude of potential research questions yet to be addressed on any one of these life history traits, each chapter focuses only on a subset of these questions. Nevertheless, I have tried to address a series of key questions which relate to these traits, and which have significance for understanding the social biology and life histories of eusocial insects in general and of bumble bees in particular. The focus of the current work on each of these traits (and others) is outlined below.

Outline of data chapters

One central, but poorly studied, issue in colony life history is how the sexual maturation of colonies is controlled, especially given that queens and workers may have different

optima over the timing of this event (insofar as it affects sex ratio). Chapter 2 examines the mechanism regulating the sexual maturation of annual eusocial insect colonies using the switch point of *B. terrestris*. Conflict over the occurrence and timing of this event may occur between workers and the queen, but information provided by workers may also be important for determining when the queen should optimally begin male production. For this reason, it is unclear whether the queen or workers have control over the switch point. I reared queens founding eusocial colonies or in experimentally enforced asocial conditions, to determine the effects on the occurrence and timing of the switch point. Thus, I use a novel treatment to establish whether queens or workers are in control of sexual maturation in an annual eusocial insect colony.

As colonies mature, their needs may change, such as the interests of colony members to control worker reproduction and defend against social parasites (which are able to exploit eusocial behaviours). Chapter 3 considers the competition point in *B. terrestris* and the effect that this colony life history event has on the behavioural response of worker-laid egg eating by colony members. This behaviour is employed to police the laying of eggs by natal workers, which by definition occurs after the competition point. However, egg-eating behaviour may also be important for policing the eggs of non-natal 'drifter' workers, which can invade the colony before the competition point. Thus, if defence against socially parasitic drifter workers has been an important driver for the evolution of worker-laid egg eating, this policing behaviour should be expected to occur before the competition point, as well as after it. In contrast, if the behaviour evolved only to police natal workers, the behaviour may be expected to occur only after the competition point. We introduced eggs laid by natal or non-natal workers to colonies both before and after the competition point, and observed the host colony responses. This experiment allowed us to describe the egg eating responses of host colonies to worker-laid eggs at different stages in colony development and to determine the likely role of social parasitism in the evolution of policing behaviour.

Changes in the external environment, e.g. climate change, may alter colony life history by affecting the behaviour and longevity of colony members, but the complexity of interactions in eusocial insect colonies make these effects difficult to predict. Chapter 4 reports the effect of ambient temperature on of a number of key colony life history traits of *B. terrestris*, and is, to the best of our knowledge, the first time such a study has been reported using complete colonies of any eusocial insect. In particular we compare the responses in colony and individual longevity, as well as colony productivity, to two different field-realistic temperatures in the laboratory. This study also allows us to test

the hypothesis that temperature is causally involved with winter activity in *B. terrestris*, which represents a deviation from the normal life history of this species. This study adds essential information about the basic responses of eusocial insect colony life history traits to different climates, and so represents a critical background study for understanding how pollinators and pests might be affected by climate change at the colony level.

The behaviours of individuals which affect colony life history are, in turn, the result of mechanisms operating at the molecular and physiological levels. For this reason, a fuller understanding of the control of colony life history requires deeper insight into genetic and physiological factors in colony members. Chapter 5 investigates the internal mechanisms by which *B. terrestris* queens cease foraging at colony foundation, i.e. the shift between solitary and eusocial life stages. The 'foraging' gene (*for*) is used as a candidate gene because previous work has indicated that this gene is linked with foraging behaviour in a number of insects. Using quantitative PCR, we measure the effects of foraging gene expression in laying and non-laying queens at various ages and under two different feeding regimes. This allows the first insight into a possible genetic correlate of colony founding behaviour. We also test the effects of these states, and of foraging gene expression, on locomotor activity, as an established proxy of foraging behaviour, to reveal which physiological states appear to be correlated with this behaviour. This experiment therefore explores a little-studied life history event for eusocial insect colonies (the cessation of queen foraging at colony foundation) by giving insight into its molecular regulation for the first time.

Chapter 2

Queen control of colony sexual maturation in the bumble bee Bombus terrestris

Abstract

In eusocial insects, inclusive fitness theory predicts potential queen-worker conflict over the timing of events in colony life history. Whether queens or workers control the timing of these events is poorly understood. In the bumble bee *Bombus terrestris*, queens exhibit a 'switch point' in which they switch from laying diploid eggs yielding females (workers and new queens) to laying haploid eggs yielding males. As the switch point represents the beginning of direct colony investment in sexual individuals, i.e. colony sexual maturation, it constitutes an important colony life history event. By rearing foundress queens whose worker offspring were removed as pupae and sexing their eggs using microsatellite genotyping, we found that queens kept in the complete absence of adult workers still exhibit a switch point. Moreover, the timing of their switch points relative to the start of egg-laying did not differ significantly from that of queens allowed to produce normal colonies. The finding that bumble bee queens can express the switch point in the absence of workers experimentally demonstrates queen control of a key life-history event in eusocial insects. In addition, we found no evidence that workers affect the timing of the switch point either directly or indirectly via providing cues to queens, suggesting that workers do not fully express their interests in queen-worker conflicts over colony life history.

Introduction

The evolution of eusocial societies represents a prime example of a major transition in evolution leading to a new level of individuality (Maynard Smith and Szathmáry 1997; Bourke 2011). For this reason, colonies of eusocial insects undergo a life history analogous in some respects to that of individual organisms (Oster and Wilson 1978). For example, in annual eusocial Hymenoptera, the change from colony growth (worker production) to reproduction (production of new queens and/or males) is a key life-history event because it represents sexual maturation at the colony level. Inclusive fitness theory predicts potential queen-worker conflict over both sex allocation (Trivers and Hare 1976) and the timing of the colony's sexual maturation (Bulmer and Taylor 1981; Bourke and Ratnieks 1999). The outcomes of such conflicts depend on which party, or parties, within the colony 'control' the relevant trait. Control here refers to any processes, either behavioural or physiological, which allow a given party to affect the trait, including responses to the external environment. By determining the primary sex ratio (Wharton et al. 2007; Aron 2012), i.e. the ratio of haploid to diploid eggs laid, queens in the eusocial Hymenoptera potentially exert considerable control in queen-worker conflicts. However, workers may also exert control, through differential rearing of offspring or, as in worker matricide, differential treatment of queens (Ratnieks et al. 2006a; Aron 2012). Additionally, the timing of colony sexual maturation may depend on queens responding to cues provided by workers. Such cues might provide information either on the colony's growth stage or on external environmental conditions such as resource availability (e.g. Shykoff and Müller 1995). They might also provide a means by which workers could indirectly manipulate the timing of colony sexual maturation in their own interests. There has been considerable focus on queen control of the primary sex ratio with respect to sex allocation (Aron 2012). But whether such control extends to colony sexual maturation, and whether workers can influence this event, either directly or indirectly, has not been experimentally tested.

The bumble bee *Bombus terrestris* is an annual eusocial insect in which colonies are founded by single queens in spring and produce first workers and then sexuals (new queens and males) before dying out in late summer (Duchateau and Velthuis 1988). Queens exhibit a well-characterized 'switch point' in which, over approximately 8 days, they change from laying exclusively diploid eggs yielding females (workers or new queens) to laying exclusively haploid eggs yielding males (Duchateau and Velthuis 1988; Duchateau et al. 2004). The switch point typically occurs 2–4 weeks after the eclosion of the first worker (emergence from pupa) (Duchateau and Velthuis 1988;

Lopez-Vaamonde et al. 2009). Along with the laying of diploid eggs yielding new queens, which tends to happen shortly beforehand, the switch point marks the colony's sexual maturation (Duchateau and Velthuis 1988; Lopez-Vaamonde et al. 2009). Since potential queen-worker conflict over sex allocation and colony sexual maturation are both present (Bulmer and Taylor 1981; Bourke and Ratnieks 1999), and since queen and workers might benefit from facultatively adjusting the timing of male production to match local conditions (Duchateau et al. 2004; Lopez-Vaamonde et al. 2009), it has been hypothesized that the social (colony) environment should affect the switch point. However, previous work has shown little evidence for this, since experimental manipulations of *Bombus* colonies, including doubling worker number in *B. terrestris*, had no significant effect on the timing of male production (Plowright and Plowright 1990; Muller and Schmid-Hempel 1992; Bloch 1999). Queens do not switch to laying haploid eggs through having exhausted their supplies of stored sperm, since post-switch queens retain plentiful, viable sperm (Greeff and Schmid-Hempel 2008).

We therefore hypothesized: (1) that *B. terrestris* queens can control the occurrence of the switch point endogenously, as Duchateau and Velthuis (1988) also suggested, and hence that queens can express the switch point in the complete absence of workers; and (2) that workers do not influence the timing of the switch point directly or indirectly. We tested these hypotheses in a single experiment in which we manipulated the presence of workers within incipient colonies and recorded the occurrence and relative timing of switch points.

Materials & Methods

Post-diapause, mated *Bombus terrestris terrestris* queens (*n* = 328) were obtained from a commercial supplier (Syngenta Bioline Bees B.V., Weert, The Netherlands) in three cohorts: cohort 1 (received between 22 April and 29 April 2010), cohort 2 (received on 17 August 2010) and cohort 3 (received on 5 May 2011); consisting of 103, 105 and 120 queens, respectively. All bees were housed at 28°C, 60% relative humidity and constant darkness, and fed *ad libitum* with freeze-dried pollen and sugar syrup obtained from Koppert B.V., Berkel en Rodenrijs, The Netherlands. On the day of receipt each queen was placed singly in a plastic box (140 × 79 × 60 mm), and on the following day a single cocoon (containing a living or frozen larva or pupa) was introduced into each box to stimulate egg-laying by the queens (Kwon et al. 2003). The cocoons were obtained from 15 mature *B. terrestris terrestris* colonies (5 per

cohort) obtained from the same supplier and were distributed randomly amongst the queens. Any adult males or workers eclosing from these cocoons were removed on the same day. For cohorts 1 and 3, cocoons were removed after 2 weeks. For cohort 2 only, living cocoons were replaced every 7 days (to avoid eclosions from them) until day 30, when all cocoons were removed. For cohort 3 only, frozen cocoons were used. All queens were checked daily for egg laying, and the date on which each queen laid her first egg was noted (n = 138 queens).

Experimental treatments

Immediately after each queen had produced her first pupa, i.e. her oldest larva had pupated, she was assigned to a 'social' or 'asocial' treatment (n = 41 queens). Within cohorts, successive queens were assigned to the treatments alternately in order to equalize the numbers of queens entering each treatment, except in the case of cohort 3 in which, to increase the sample size of asocial queens, each assignment of a queen to the social treatment was followed by the assignment of the next two queens to the asocial treatment. On being assigned to a treatment, individual queens were moved to larger wooden boxes (300 × 200 × 170 mm) with clear Perspex lids.

The social treatment allowed queens to raise a colony in the normal way. Asocial queens were inspected each day for the presence of pupal cocoons, and any present were removed and discarded. The asocial treatment therefore allowed queens to lay eggs but prevented them from being exposed to any adult offspring. To control for effects of disturbance stemming from the removal of cocoons, cocoons were also removed from social queens and then returned. In cohorts 1 and 2, on each occasion that cocoons were removed from an asocial queen, an approximately equal number of cocoons was removed from her paired social queen (i.e. the next queen that had entered the social treatment) and then returned. In cohort 3, on each occasion that cocoons were removed from an asocial queen, approximately half that number was removed from the paired social queen and then returned. Because each social queen was paired with two asocial queens in cohort 3, this procedure ensured that the number of cocoons removed and replaced per unit time was equalized across treatments in each cohort. For both treatments, removal of cocoons involved, when necessary, the cutting away of the cocoons from any attached brood items, to ensure that only cocoons were removed.

Egg sampling and observations

Every new egg-cell produced by queens in both treatments was removed, censused and sampled for some of its eggs. In each egg cell, if five or more eggs were present, two were removed; if 2–4 eggs were present, one was removed, and if only one egg was present, it was not removed. This procedure allowed the sampling of eggs for microsatellite-based sexing while ensuring that queens were not deprived of all their eggs. After egg removal, egg cells were resealed and replaced in the boxes. All removed eggs (*n* = 1352) were frozen for sexing.

In *B. terrestris*, workers can produce male offspring but the first worker-laid haploid eggs are nearly always laid at a point in the colony cycle (the 'competition point') that follows the switch point, with worker-laid haploid eggs appearing before queen-laid haploid eggs only rarely (Duchateau et al. 2004; Lopez-Vaamonde et al. 2009). Nonetheless, daily behavioural observations on colonies of social queens allowed us to check for worker-worker and worker-queen aggression, which are indicative of a colony having passed the competition point (Duchateau and Velthuis 1988; Duchateau 1989).

Colonies were terminated following either the death of the queen or first male eclosion, or, if these events had not occurred, 4–6 months after the beginning of the experiment.

Genotyping and sexing of eggs

Sampled eggs were sexed using genotyping at five polymorphic microsatellite loci: *B10*, *B11*, *B118*, *B121* and *B124* (Estoup et al. 1995; Estoup et al. 1996). DNA was extracted from the eggs using the HotSHOT protocol (Truett et al. 2000). DNA at the five loci was amplified using multiplex PCR and then genotyped. PCR mixtures consisted of 1 µl of DNA extract (allowed to evaporate for 30–60 min beforehand), 1 µl of QIAGEN multiplex mix and 1 µl of primer mix. Reaction mixtures were covered by mineral oil to prevent evaporation and run in a MJ Research Thermal Cycler PTC240 Tetrad 2 for 15 min at 95^{o}C (for activation of Taq polymerase), then for 35 cycles of 30 s denaturing at 94^{o}C, 90 s annealing at 57^{o}C and 60 s extension at 72^{o}C, and then for 30 min at 60^{o}C (for completion of newly synthesised strands). Primer concentrations used were as follows: *B10*, 0.336 µM; *B11*, 0.084 µM; *B118*, 0.120 µM; *B121*, 0.072 µM; *B124*, 0.120 µM. PCR products were genotyped using LIZ 500 marker dye in an ABIPrism 3730 capillary sequencer. Resultant electropherograms were interpreted and scored blindly with respect to date of sampling and treatment using GENEMAPPER v.4 (Applied Biosystems). GENALEX 6 (Peakall and Smouse 2006)

was used to test for Hardy-Weinberg equilibrium and to calculate heterozygosities, the input data being genotypes of queens alone.

After the scoring was completed, egg genotypes were used to deduce the likely genotypes for each queen and each queen's mate (e.g. an allele common to all diploid eggs of a single queen could be identified as the queen's mate's allele), with the queen's genotype then being checked by genotyping of the queens. All queens in each treatment were genotyped twice using the same methods as used for eggs, following DNA extraction from a single leg (n = 41 queens, with 5 yielding incomplete genotypes). Samples in which at least one locus could not be scored were omitted from analyses (n = 388 eggs). Samples with alleles scored at any locus that could not be accurately reconciled to queen and sibling genotypes, including eggs from queens laying too few eggs, were also omitted from analyses (n = 123 eggs). A random subset of 31 eggs was re-genotyped after repeat PCR to estimate the genotyping/scoring error rate. Genotyping/scoring errors were detected as differences between alleles scored in each of these eggs, and as differences between alleles scored in positive controls (4 eggs of known genotype run on every plate), with any missing scores ignored.

In haplodiploid Hymenoptera, eggs appearing homozygous at multiple polymorphic loci will, with high likelihood, be haploid (Ratnieks and Keller 1998; Lopez-Vaamonde et al. 2003). If at a given locus the queen's mate bore an allele (paternal allele) different from both of the queen's alleles (e.g. mating type, AB × C or AA × B), the chance of diploid offspring at that locus being homozygous was zero; loci of this type were regarded as 'informative loci'. If the paternal allele was the same as one of the queen's alleles (e.g. AB × A), half the diploid offspring would be homozygous; such loci were regarded as 'semi-informative'. Loci in which the queen was homozygous and the paternal allele was the same (e.g. AA × A) were regarded as 'uninformative'. In principle, a single informative locus would be sufficient to identify an egg as diploid or haploid. However, we adopted the conservative approach of using either two informative loci or one informative and two semi-informative loci as a minimum requirement for an accurate classification. A queen's switch point was defined as the number of days between her first egg and her first observed haploid egg (as inferred from the egg genotypes). A previous study has shown that the switch point estimated from genotyping eggs correlates highly with that estimated by back-calculation from the date of eclosion of the first adult male (Lopez-Vaamonde et al. 2003).

Because *B. terrestris* workers can be reproductive, haploid eggs could, in principle, have been queen- or worker-produced. In social queens, we therefore checked for worker egg-laying by inspecting haploid eggs for the presence of the paternal allele, since, at any locus, workers transmit this allele to 50% of their sons. We used these genetic data in combination with behavioural evidence of the occurrence or non-occurrence of the competition point (see 'Egg sampling and observations' above) to determine whether worker egg-laying had occurred in colonies of social queens.

Statistical analysis

A two-way ANOVA was used to test whether the timing of the switch point was affected by treatment (social versus social conditions), cohort or treatment-cohort interaction. Model simplification was used by removing non-significant terms, starting with the interaction term, and then testing each of the other two terms independently, to find the minimum adequate model. Models conformed to the assumptions of normally-distributed residuals and homoscedasticity (after $\log_{10}$ or reciprocal transformation of switch point) as tested using Shapiro-Wilk tests and Levene's test for homogeneity of variances, respectively.

A random re-sampling method was used to investigate the effect on the switch point estimates of the smaller number of eggs sampled and genotyped from asocial queens relative to the corresponding number from social queens (fig 2.1). Specifically, we tested whether switch points obtained from asocial queens differed significantly from simulated ones obtained from sampling an equivalent number of eggs from social queens. We first selected a random social queen, and randomly sampled eggs from her (with replacement) equal to the total number of genotyped eggs taken from a randomly selected asocial queen within each 10-day period. A switch point was then calculated for these sampled eggs by finding the 10-day period in which the first haploid egg occurred. This process was carried out 1000 times, with replacement, and the distribution of simulated switch points was compared with the actual distribution of switch points found in asocial queens using a Wilcoxon rank sum continuity test (since the distribution of switch points for both groups was non-normal). All simulated queens showing no switch point (n = 823) were excluded from the comparison, as they were equivalent to non-switching queens.

We also conducted a power analysis to calculate the minimum detectable difference in the switch points of our samples of social and asocial queens. Specifically, we calculated the minimum difference between means needed to show a significant effect

of treatment in our ANOVA. This was done by experimentally adding (or subtracting) a set number of days to each value within a single treatment group and performing the ANOVA (using a model with the treatment term only) using these modified values to test whether such changes were significant at $\alpha = 0.05$. This method assumed that the observed standard deviations of switch point time within each treatment were the true population standard deviations. Since this method is not always reliable (Colegrave and Ruxton 2003), 95% confidence intervals between treatments were also calculated using the untransformed switch point data.

Some queens produced no haploid eggs and we hypothesized that this was because they ceased egg production before reaching their switch point. To test this, the date on which the last successfully genotyped diploid egg was laid by each such queen was compared with the switch point of switching queens using a Welch's *t*-test (after $\log_{10}$ transformation). In this analysis, to increase statistical power, we pooled social and asocial queens within non-switching and switching categories (separating them out yielded differences between means in the same direction). The non-switching category comprised 3 social and 9 asocial queens (see 'Results'). The number of eggs laid by queens before the switch point was compared between treatments using a Welch's *t*-test.

All distributions were checked for normality (Shapiro-Wilk tests), and non-parametric tests were used for those not conforming. For all *t*-tests, homogeneity of variances was checked using Fisher's *F* test (where this did not hold, a Welch's *t*-test was used, i.e. assuming unequal variances between samples). All statistical tests were performed using R (R Development Core Team 2011). Unless otherwise stated, all means are expressed ± 1 SD.

Results

Genotyping

The total numbers of alleles detected per locus were 20, 9, 10, 5 and 10 for *B10*, *B11*, *B118*, *B121* and *B124*, respectively. All loci except *B118* were found to be in Hardy-Weinberg equilibrium, with observed heterozygosity values of 0.66, 0.88, 0.16, 0.78 and 0.79 for loci *B10*, *B11*, *B118*, *B121* and *B124*, respectively. The mean per locus error rate in initial allele identification obtained from scoring both random re-types and positive controls ($n = 70$) was 3.6% (range across loci, 0.0–7.9%). This error was unlikely to have affected the final sex allocated to each egg because, as described in

the ‘Materials & Methods', all egg genotypes that could not be reconciled with queen and sibling genotypes were discarded.

Sample sizes

Of the 328 queens in the experiment, 138 (42%) laid at least one egg, of which 41 queens produced at least one pupa (28% of egg-layers). Of these 41 queens, 17 were assigned to the social treatment (five from cohort 1, six from cohort 2 and six from cohort 3) and 24 were assigned to the asocial treatment (five from cohort 1, five from cohort 2 and fourteen from cohort 3). Mean (± SD) total numbers of eggs laid were 208.6 ± 202.1 for social queens (n = 17) and 52.8 ± 51.6 for asocial queens (n = 24), over means (± SDs) of 65.5 ± 23.5 days and 42.5 ± 19.5 days, respectively. In total, 978 eggs and 374 eggs were sampled and genotyped from social queens (n = 17) and asocial queens (n = 24), respectively.

In six queens (1 social and 5 asocial), few eggs (<6) were sampled and they gave conflicting information for deducing the genotype of the queen's mate, and so, as described in ‘Materials & Methods’, these queens were omitted from the analysis. Following this and the other exclusions described in ‘Materials & Methods’, sample sizes of eggs that were successfully genotyped and whose genotypes could be reconciled with those of the mother queen and her other eggs became 634 and 207 for social queens (n = 16) and asocial queens (n = 19), respectively.

In a further five queens (2 social and 3 asocial), haploid eggs were laid before the first pupa was produced, i.e. before assignment to treatments for those queens. The reason for such early haploid egg production was unknown, although sporadic occurrences of males in the first brood have previously been recorded (Alford 1975). These queens were omitted since haploid egg production at these times could not have been a result of treatment.

In the 30 remaining queens (14 social and 16 asocial), each had either at least two informative loci or at least one informative locus and two semi-informative loci. Of these, 11 social queens and 7 asocial queens produced at least one haploid egg. In colonies of eight of these social queens, the behavioural observations showed no evidence of the competition point having occurred in the sampling period and no paternal alleles (indicative of worker egg-laying) were identified in haploid eggs. In colonies of each of the three remaining social queens, behavioural observations did show evidence of the competition point having occurred and paternal alleles were

identified in haploid eggs. In one of these (SM279; fig. 2.1), only 1 of 13 haploid eggs bore the paternal allele and this was the last-laid haploid egg. In the second queen (SM190; fig. 2.1), the first haploid egg laid bore a paternal allele, but the next could be confidently identified as queen-laid (from the combined probabilities of its loci bearing a paternal allele if it were worker-laid being low, i.e. $p = 0.033$); this egg therefore defined SM190's switch point. In the third queen (SM142), the first haploid egg also bore a paternal allele, but subsequent haploid eggs could not be confidently identified as queen-laid (from the combined probabilities of their loci bearing a paternal allele if they were worker-laid being too high, i.e. $p > 0.05$). This queen was therefore excluded from further analyses. This resulted in a sample size of 10 social queens and 7 asocial queens included in the final analyses as 'switching queens'. In these switching queens, the time of first cocoon removal (and hence assignment to treatment) did not significantly differ between treatments (social queens mean ± SD: 28.8 ± 8.36 days after first egg; asocial queens mean: 25.1 ± 7.5 days after first egg; Wilcoxon rank sum test, $W = 46.5$, $n = 17$, $p = 0.281$).

Occurrence and timing of the switch point

Amongst switching queens, the mean (± SE) switch points of social and asocial queens were 53.0 ± 2.65 and 56.0 ± 5.41 days, respectively (fig. 2.1a, 2.2). These switch points did not differ significantly (two-way ANOVA, $F_{1, 15} = 0.16$, $p = 0.697$). Furthermore, there was no significant effect of either cohort ($F_{2, 14} = 1.71$, $p = 0.216$) or treatment-cohort interaction ($F_{2, 11} = 3.17$, $p = 0.082$; fig. 2.3) on switch point. Switch points of the asocial queens did not differ significantly (Wilcoxon rank sum continuity test, $W = 775$, $n = 177$, $p = 0.239$) from simulated switch points calculated by re-sampling eggs from social queens at sample sizes equivalent to those obtained in asocial queens. This showed that lower sample sizes of eggs for asocial queens (fig. 2.2) did not bias the switch point estimates. However, the power analysis (see *'Materials & Methods'*) showed that the minimum detectable difference in the switch points of our samples was 11–12 days. Similarly, the 95% confidence interval for the difference between the asocial mean switch point and the social mean switch point was -14.7 to 8.7 days.

Queens that failed to exhibit a switch point laid their last diploid egg significantly earlier than the switch point of switching queens (means ± SE: 43.0 ± 3.89 v. 54.2 ± 2.64 days after first egg, respectively; Welch's *t*-test, $t_{15} = 2.36$, $p = 0.032$), suggesting that non-switching queens failed to switch because they had stopped laying eggs before the

switch point was reached. Amongst switching queens, social queens laid significantly more eggs (over 3 times more) before the switch point than asocial queens (Welch's *t*-test, $t_{11} = 4.71$, $p < 0.001$; fig. 2.1b). Asocial queens almost certainly laid fewer eggs through lacking resources and aid supplied by workers, and this, combined with earlier cessation of egg-laying leading to a lower probability of switching, would account for the smaller proportion of asocial queens that exhibited a switch point relative to social queens.

In one switching asocial queen, an adult eclosed from the cocoon that was added to stimulate queen egg-laying and was removed on the same day. In the six other switching, asocial queens, the added cocoon was removed before eclosion. Therefore, all seven asocial, switching queens exhibited a switch point without ever being exposed to their adult offspring, and six of the seven exhibited a switch point without ever experiencing contact with another adult bee following the start of the experiment.

Finally, in switching social queens, the median 'sharpness' of the switch point (the median interval between the switch point, i.e. date of first observed haploid egg, and the date of the last diploid egg laid) was 10.5 days ($n = 10$). This value is comparable with the value of 8 days reported by Duchateau et al. (2004), especially if it is borne in mind that the two estimates were made using different methods, since Duchateau et al. (2004) used the method of back-calculation from the date of eclosion of the first male.

Discussion

We found that queens of the bumble bee *Bombus terrestris* reared in asocial conditions, in which they were never exposed to their adult worker offspring, switched to laying haploid, male eggs as did social, control queens allowed to produce adult worker offspring in the normal way. This result experimentally demonstrates that the switch point, which represents a key life-history event in colony development, can be controlled by queens endogenously. Moreover, we found no significant difference in the timing of the switch point between social queens and asocial queens. However, our data do not preclude an influence of workers on the timing of the switch point within the limits specified by the power analysis. In addition, there may have been a differential effect of treatment on switch point timing in response to cohort, since the treatment-cohort interaction was close to significance. This may be a result of alternative queen rearing treatment in cohort 2 (using live pupae), but the sample sizes for each cohort were too small any firm conclusions based on individual cohorts. Nonetheless, we found no evidence that workers exert a large influence over colony sexual maturation

either directly or indirectly, suggesting that workers do not express their interests fully in queen-worker conflicts over colony life history. This evidence is complemented by the earlier study of Alaux et al. (2005), which showed that queens can also influence the onset of new queen production, suggesting that queens predominantly possess power over both components of colony sexual maturation.

It is not clear whether queen control of the switch point has arisen because there are phylogenetic constraints on which kind of mechanisms can evolve or because it is the most adaptive solution for queens. In relation to other *Bombus* species, the present finding of queen control over the switch point is consistent with previous data showing that haploid eggs can be laid before the emergence of the first workers in colonies of *B. lucorum* and *B. terricola* (Müller et al. 1992). This suggests that queen control of the switch point may be widespread in *Bombus*. However, the extent to which many other *Bombus* species exhibit a clear switch point in the sense of *B. terrestris* is not clear (although a similar switch point is known in at least *B. terricola*; Plowright and Plowright 1990). For example, a reversion to the production of workers must occur in species with perennial nests such as *B. atratus* (Garofalo 1974), and even in annual species the transition to the exclusive production of haploid eggs might progress slowly or be incomplete. Further study of other species will be necessary to be determine whether queen control over the onset of male production is ubiquitous across bumble bees.

Since some solitary bees have been shown to exhibit, during their life history, a switch from laying female to laying male eggs (Stark 1992; Seidelmann et al. 2010), it is possible that the endogenous mechanism controlling the switch point in bumble bee queens has been inherited from their solitary ancestors. It is possible that worker influence on the switch point may be difficult to evolve, even if it would be adaptive to both parties for the queen to account for colony-size or seasonal information derived from workers. However, an endogenous mechanism of determining the switch point may alternatively be adaptive to *B. terrestris* queens, because it allows them to exercise control in kin-selected conflicts with workers over sex allocation (Brown et al. 2003) and colony sexual maturation (Bulmer and Taylor 1981; Müller et al. 1992; Bourke and Ratnieks 1999). It does not follow from endogenous queen control of the switch point that the switch point is entirely unresponsive to external cues. For example, Duchateau *et al*. (2004) found that *B. terrestris* queens undergoing longer periods of diapause exhibited earlier switch points, suggesting that queens use 'personal' cues stemming from their pre-founding or founding experience and/or their own quality to modulate the timing of the switch point.

We hypothesize that queens use such personal cues to initiate an internal interval timer (Paul et al. 2008; Visser et al. 2010) that 'counts down' to the switch point. This hypothetical timer appears to be independent of periodism in light levels and temperature, since queens were kept in darkness at constant temperature. Queens do not lay a standard number of diploid eggs before switching to laying haploid eggs, since we found that social queens laid significantly more eggs than asocial queens before switching even though they switched at the same relative date. Overall, the proximate mechanism underlying endogenous queen control of the switch point in *B. terrestris* is unknown and deserves future investigation.

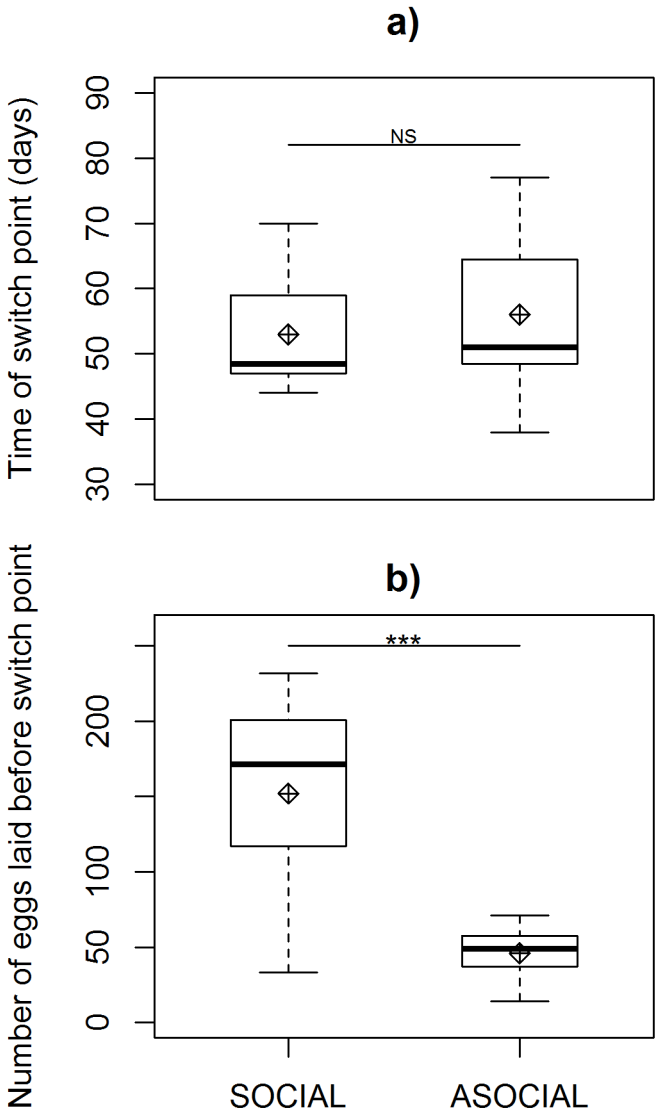

Figure 2.1 (a) Time of the switch point (days from queen's first egg) and (b) number of eggs laid before the switch point in *Bombus terrestris* queens in social (*n* = 10) and asocial (*n* = 7) treatments. Diamonds, means; thick horizontal bars, medians; boxes, interquartile range; whiskers, range. (a) NS, not significant (ANOVA); (b), ***, $p < 0.001$ (Welch's *t*-test).

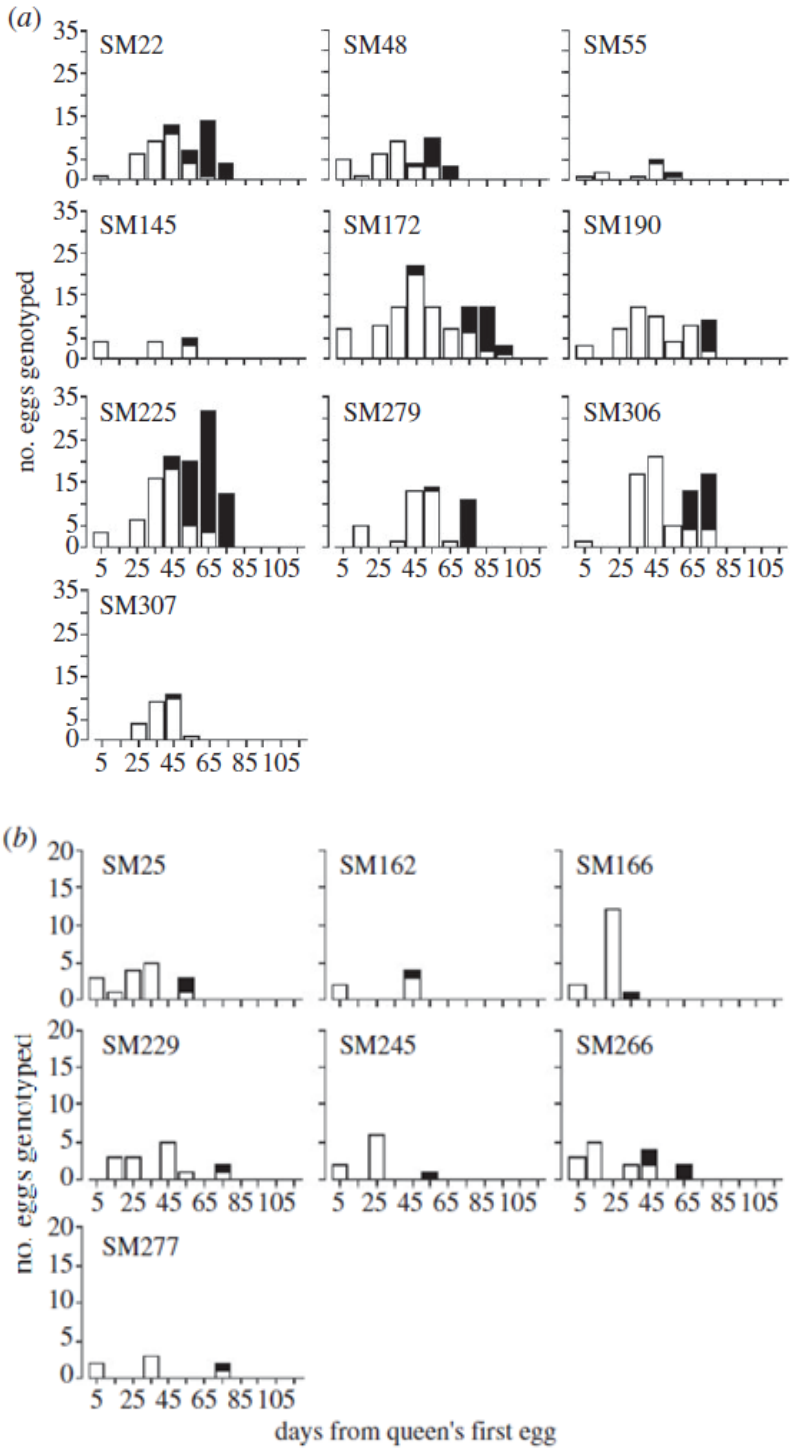

Figure 2.2 The numbers of sampled diploid and haploid eggs laid by *Bombus terrestris* queens over time in (a) social and (b) asocial treatments (*n* = 10 and 7 queens, respectively). Each plot represents a separate queen (id code in upper left corner). White shading, diploid eggs; black shading, haploid eggs; total heights of bars, number of eggs genotyped in each time bloc.

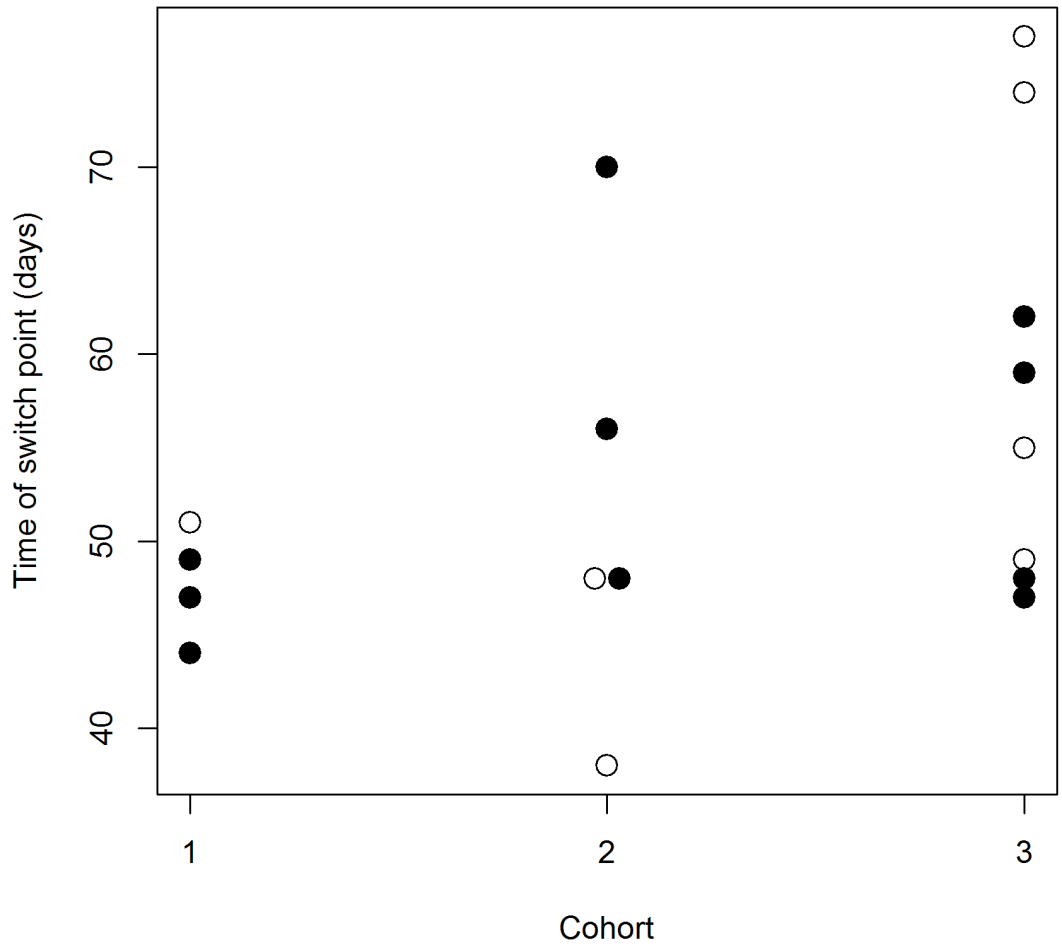

Figure 2.3 Time of the switch point (days from queen's first egg) in *Bombus terrestris* queens according to cohort (groups of queens received at separate times). Queens in social (*n* = 10; black circles) or asocial (*n* = 7; white circles) treatments.

Chapter 3

Worker policing of eggs and intraspecific social parasitism in the bumble bee Bombus terrestris

Abstract

Eusocial insect colonies are vulnerable to exploitation by natal reproductive workers and also by intra-specific parasites such as drifting workers, which lay eggs in the host colony. Egg-eating is a well studied mechanism of host colonies policing the first of these, but it may also be a way of policing the second, and the evolutionary significance of these two functions in the origin of policing behaviour is not known. Social bumble bee colonies exhibit a shift to worker egg-laying at a specific point in colony development (the competition point), but also experience intraspecific parasitism by non-natal drifting workers throughout. Since these drifters are capable of laying eggs before the host colony's competition point, a failure to police worker-laid eggs at this time would reveal that drifter workers have not been of high importance in the evolution of policing. We introduced artificial egg cells containing eggs laid by either natal or non-natal workers into host colonies of the social bumble bee *Bombus terrestris* both before and after their competition points, and observed colony responses to these cells. No significant difference was found in the egg-eating rate or egg cell fate according to either egg origin or whether the host colony had passed the competition point. This suggests that, in contrast to other social insects studied, bumble bees cannot discriminate worker-laid eggs based on colony origin. However, the results are consistent with a role of intraspecific social parasitism in the evolution of policing, because worker-laid eggs were eaten at high frequency (ca 75% in 20 hours) before the competition point, i.e. before natal workers in the host colony began egg-laying.

Introduction

The evolutionary success of the eusocial insects lies partly in a division of labour between reproduction and brood rearing, allowing non-reproductive individuals to rear the offspring of their nestmates cooperatively (Oster and Wilson 1978; Bourke 2011). However, this decoupling of brood production and brood rearing also makes eusocial insect colonies vulnerable to exploitation (Oldroyd 2013). One such form of exploitation is rogue reproduction by natal workers, which in the eusocial Hymenoptera (ants, bees and wasps) are female. Although workers are generally sterile or refrain from reproduction, they can sometimes be capable of producing males from unfertilised eggs if it coincides with their inclusive fitness interests (Trivers and Hare 1976; Bourke and Franks 1995; Heimpel and de Boer 2008). This may occur in workers if they are more closely related to their own offspring than the offspring of others, and if they are physiologically capable of producing a sufficient number of offspring relative to the number that their relatives (e.g. colony queens) could produce; i.e. in accordance with kin selection theory (Hamilton 1964; Dawkins 1976; Trivers and Hare 1976). The production of eggs by natal workers may not be tolerated by nestmates for several reasons: 1) because the relatedness between these eggs and nestmates may be low (Ratnieks 1988; Bourke and Franks 1995; Wenseleers and Ratnieks 2006a); 2) because worker reproduction could detract from effective division of labour (Ratnieks 1988; Wenseleers et al. 2005; Ohtsuki and Tsuji 2009; Teseo et al. 2013); and 3) because eggs laid by workers, as males, will not develop into workers, which are often more beneficial to nestmates (Foster and Ratnieks 2001b; Wenseleers and Ratnieks 2006a). One key behavioural mechanism for queens and workers to police egg-laying by rivals is egg-eating, which has been observed across the eusocial hymenoptera as a defence against egg-laying by natal workers (Van Doorn and Heringa 1986; Ratnieks and Visscher 1989; Monnin and Peeters 1997; Foster and Ratnieks 2001a; Wenseleers and Ratnieks 2006a; Zanette et al. 2012).

A second form of exploitation in eusocial insects is social parasitism, whereby colonies are fooled into rearing the offspring of a parasite (Field 1992; Beekman and Oldroyd 2008). Social parasitism is especially prevalent among closely related species (Emery's rule; see Buschinger 2009) and in conspecifics (e.g. Field 1992; Foitzik and Heinze 2000; Lopez-Vaamonde et al. 2004; Nanork et al. 2005; Hartel et al. 2006; Beekman and Oldroyd 2008). Accordingly, policing by egg-eating can also act as a defence against offspring produced by social parasites (Beekman and Oldroyd 2008), which are detrimental to all (non-parasite) within-colony parties because they represent a waste

of colony resources on individuals which may go on to propagate genes from a rival genotype. However, the relative importance of these two functions of egg-eating in eusocial insects (i.e. policing against eggs laid by natal workers and against those laid by social parasites) is not known. In particular, it is not clear which of these functions was responsible for the evolutionary origin of egg-eating in eusocial insect lineages (Pirk et al. 2007; Beekman and Oldroyd 2008; Zanette et al. 2012).

In order for an individual in a colony to preferentially police specific types of eggs (i.e. eggs which it would be in their fitness interests to destroy), it must be able to discriminate between eggs of different origins. From the perspective of an egg which would, if detected, be preferentially eaten, it would instead be beneficial for it to evade discrimination. This creates the conditions for an evolutionary arms race, with eggs and egg-layers, such as reproductive workers and social parasites, on one side being selected to avoid detection, and natal adults on the other side being selected to discriminate them from other eggs. This is similar to the phenomenon of evasion by adult social parasites (Lenoir et al. 2001; Buschinger 2009), but the discrimination of eggs has been much less studied in eusocial insects (Chernenko et al. 2011). Discrimination of eggs in the eusocial Hymenoptera (ants, bees and wasps) has been, in various contexts, largely ascribed to variation in egg surface chemistry (Endler et al. 2004; van Zweden et al. 2009b), although a recent study has shown that egg cells (sealed wax cups in which eggs are contained) may also be important for egg discrimination in bumble bees (Zanette et al. 2012). Discrimination of non-natal worker-laid eggs has been found in *Formica* ants (Helantera and Sundstrom 2007; Meunier et al. 2010; Chernenko et al. 2011). In honey bees (*Apis mellifera*), workers are also capable of discriminating natal and non-natal eggs, with eggs laid by non-natal queens or workers being removed faster than eggs laid by nestmates (Pirk et al. 2007). However, some eggs of parasitic or anarchistic honeybee workers have been shown to escape policing (Oldroyd and Ratnieks 2000; Martin et al. 2002).

Social bumble bees are a well studied system within the eusocial Hymenoptera exhibiting both worker reproduction and intra-specific social parasitism. Bumble bee colonies possess an annual colony cycle, and towards the end of colony life exhibit a ‘competition point’, a life history event after which natal workers begin to lay their own eggs, and aggression between individuals usually ensues (Duchateau and Velthuis 1988; Bourke and Ratnieks 2001; Amsalem et al. 2009). The competition point is also the time at which policing normally begins, consisting of egg-eating of worker-laid eggs by both workers and the colony queen, and resulting in 100% of queen-laid eggs, but

less than 10% of worker laid eggs, surviving to 2 hours (Zanette et al. 2012). As in some other eusocial insects (Beekman and Oldroyd 2008), bumble bees exhibit intra-specific social parasitism in the form of reproductive drifting workers. These drifting workers, i.e. workers relocating to non-natal colonies, have been documented in bumble bee colonies in both semi-natural (Birmingham et al. 2004; Lopez-Vaamonde et al. 2004; Lefebvre and Pierre 2007) and wild settings (Takahashi et al. 2010; O'Connor et al. 2013; table 3.1). Occasionally, this behaviour may result from maladaptive orientation errors (Lopez-Vaamonde et al. 2004). However, marking nest entrances with conspicuous patterns or landmarks does not significantly reduce drifting (Birmingham and Winston 2004), suggesting that orientation error is not likely to be the major cause of this phenomenon (Beekman and Oldroyd 2008). Since workers have been demonstrated to lay eggs in non-natal nests after drifting (Lopez-Vaamonde et al. 2004; Takahashi et al. 2010; O'Connor et al. 2013), social parasitism is likely to be the dominant cause of drifting worker behaviour.

Because natal worker egg-laying in bumble bee colonies only occurs after the competition point, this system may help to reveal the relative evolutionary importance of policing eggs laid by natal versus non-natal (i.e. socially parasitic) workers. For example, since drifter workers are capable of laying eggs before the competition point (Lopez-Vaamonde et al. 2004), it is possible that drifter-laid eggs may be able to avoid policing if they are present in a host colony before this time. If this is true it would suggest that policing is primarily employed as a defence against laying by natal workers, and that defending against eggs laid by socially parasitic workers has not been of great evolutionary importance. It is also possible that eggs laid by non-natal and natal workers are treated differently, for example, the eggs of non-natal workers could be policed at all stages of colony development, whereas policing against the eggs of natal workers may only occur after the competition point. However, whether individuals in bumble bee colonies can discriminate between natal and non-natal worker-laid eggs is not currently known. In this study we employ a factorial design, introducing eggs with either a natal or non-natal origin to colonies of the bumble bee *Bombus terrestris*, both before and after the competition point. We test the hypotheses: 1) that eggs laid by non-natal workers are more vulnerable to policing than eggs laid by natal workers, and 2) that worker-laid eggs introduced after the competition point are more vulnerable to policing than worker-laid eggs introduced before the competition point.

Table 3.1; Demographics of drifting workers in previous studies. Colonies with queen unless otherwise stated. Reported values for final three columns shown as mean proportions unless otherwise stated. Means shown ± SDs where known. U = value unknown or otherwise unreported in publication.

Study	Colony type	Colony stage	Species	Number of colonies	Proportion of workers dispersing from colony as drifters*	Proportion of adults in host colony with non-natal origin†	Proportion of males derived from non-natal workers
Lopez-Vaamonde *et al.* 2004	semi-wild	pre-CP	*B. terrestris*	32	U	frequency = 9.0 ± 5.1	0.019
Lopez-Vaamonde *et al.* 2004	semi-wild	post-CP	*B. terrestris*	32 (same colonies as above)	U		0.021
Lopez-Vaamonde *et al.* 2004	semi-wild	queenless	*B. terrestris*	32 (same colonies as above)	U		0.034
Birmingham *et al.* 2004	green-house (2001)	30-100 workers	*B. occidentalis*	30	range = 0.003 to 0.348	0.072 ± 0.018	U
Birmingham *et al.* 2004	green-house (2002)	U	*B. occidentalis*	32	range = 0.005 to 0.124		U
Birmingham *et al.* 2004	green-house (2001)	30-50 workers	*B. impatiens*	12	range = 0.001 to 0.122		U
Lefebvre & Pierre 2007	green-house	34-53 workers	*B. terrestris*	9	0.198 ± 0.005	0.206 ± 0.002	U
Takahashi *et al.* 2010	wild	mature (late summer)	*B. deuteronymus*	11	U	0.013	0.066
O'Connor *et al.* 2013	wild	mature (late summer)	*B. terrestris*	14	U	frequency < 1	0.03

* Range of values shown where proportion unknown. † Mean frequency per colony shown (± SD) where proportion not known. CP, competition point.

Materials & Methods

Ten mature, pre-competition point *Bombus terrestris terrestris* colonies were supplied by a commercial rearing company (Syngenta Bioline, Weert, Netherlands) in late 2009. Colonies were contained in plastic nest boxes provided by the supplier and maintained in a dark controlled environment room at 28^{o}C and ca. 50% relative humidity for the duration of the experiment; work was conducted under red light, which is invisible to bees. In order to obtain worker-laid eggs, workers and brood cells were removed from

each colony on 24 November 2009 and placed into isolated plastic boxes (140 x 79 x 60mm). Three boxes were made per colony, with each box containing 3 workers and ca. 5 brood cells. In order to maintain at least 3 workers per box, dead workers were replaced by adding extra workers from the original supply colony (numbers were occasionally higher than 3, due to worker eclosion from brood cells). Both the colonies and isolated workers were fed *ad libitum* with pollen and sugar syrup ('Attracker'; Koppert Biological Systems, Berkel en Rodenrijs, Netherlands) throughout the experiment. When at least one box of isolated workers from each colony had begun to lay eggs (3 December; 9 days after removal), we began the egg introductions, with this day classed as the beginning of the experiment.

Egg introductions

Egg introductions were conducted in order to test the response of colonies to eggs laid by either natal or non-natal workers. For each introduction, one of us (JH) introduced the contents of one or more egg cells taken from the boxes of isolated workers to a manually-constructed artificial egg cell (mean ± SD = 6.3 ± 1.5 eggs supplied to each new egg cell). In order to standardise across treatments, artificial egg cells were formed from wax taken from an empty cell in the receiving colony on the day of the introduction, and approximately mimicked the size and shape of *B. terrestris* egg cells. The isolated workers from which the eggs were taken were originally from either the receiving colony (natal treatment) or another colony (non-natal treatment). In order to keep the number of eggs originating from each colony approximately equal, colonies were grouped into pairs at the start of the experiment; where possible, non-natal egg provided to a focal colony in an introduction were sourced from isolated workers originating from the colony paired with the focal colony (109 of 119 introductions). On dates when isolated workers from a paired colony failed to provide sufficient eggs for introduction to a focal colony, eggs were instead taken from isolated workers from another colony. Where this was not possible (due to insufficient eggs), no introduction was performed on the focal colony that day. While moving eggs to the artificial egg cell, care was taken to ensure that wax from the worker-made egg cell did not contaminate the wax used to construct the artificial egg cell, and that both the cell and eggs only came into contact with the tools used (cocktail sticks and forceps) and nitrile gloves. After each artificial egg cell (containing the eggs) had been sealed, the cell was then placed in the receiving colony and fixed on top of brood cells in a visible position. As far as was possible, two-day periods were used for introductions, in which a colony would receive egg cells in one treatment on the first day, and in the other treatment on the

second day (in a randomly determined order). This helped to ensure that treatments were equally represented across time for each colony. For one colony, five natal introductions and six non-natal introductions were conducted; in the nine remaining colonies, six introductions in each treatment were conducted. No more than one introduction per day was conducted in any one colony, and all introductions were performed between 1400 and 1800.

Competition points

Since we predicted that the response of the colony to eggs would differ according to whether or not the colony had passed the competition point, the competition status (i.e. whether before or after competition point) of each colony was assessed regularly (every 1-2 days), until it was clear that the colony had passed the competition point. Following Duchateau et al. (2004), a colony was said to have passed the competition point when at least one of the following criteria was observed: 1) multiple open egg cells; 2) egg-eating by queen or workers; 3) aggression between queen and workers; or 4) egg-laying by workers. The opening of, or egg-eating within, introduced experimental egg cells was not used to class a colony as having passed the CP (since such behaviours may have been the result of the experimental treatment and did not necessarily demonstrate egg-laying by workers in the receiving colony).

Assessment of egg cell fate

After each introduction was conducted, the introduced (artificial) egg cell was observed at 30 minute-intervals over 3 hours to check whether or not the cell had been permanently opened, with no eggs remaining. These observations were carried out blindly by a second investigator (TN), i.e. who was naive as to the treatment of the cell. After ca. 20 hours, the introduced egg cell was assessed as destroyed (open and with no eggs remaining) or not destroyed, and, if not destroyed, it was removed from the colony and dissected so that the number of eggs remaining could be counted. Occasionally, it was observed that eggs had been laid in the introduced egg cell, and this was also shown by data from digital filming (see *Results*). Although it was not possible for the investigator to discriminate between such eggs and eggs present when the egg cell was introduced, egg-laying in the introduced egg cells was unlikely to have affected the conclusions because a) it was relatively rare (occurring in 2 of 15 filmed introductions; see *Results*), and b) a relatively large proportion of egg cells were

destroyed (see *Results*), so these would have been scored as destroyed even if they had contained eggs laid since introduction of the egg cell.

Video observations

In addition to the observations described above, 15 introduced egg cells (3 natal, pre-competition point; 2 non-natal, pre-competition point; 4 natal, post-competition point; 6 non-natal, post-competition point) were also filmed for 10 hours (or until the cell was destroyed) using digital cameras (Sony) so that the causes of any changes in egg number could be identified. The recording began within 30 minutes of the egg cell introductions. Seven introductions were filmed at the start of the experiment (day 1 or 2), and eight introductions were filmed at the end of experiment (day 15 or 16). Each colony was filmed at least once (but no more than twice). The video recordings were used to identify instances of egg cell opening, egg-eating or egg-laying with regard to the introduced egg cells. This allowed us to confirm that egg-eating was responsible for the reduction in egg numbers in introduced egg cells and also to estimate the extent of egg-laying (if any) into introduced egg cells. The caste (queen or worker) of individuals performing these actions could also be ascertained from these observations. Additionally they allowed us to ensure that workers were always capable of opening the introduced egg cells (since survival of all eggs could conceivably have been due to an inability of workers to access them).

Statistical Analyses

The effect of treatment (egg origin) and competition status on the rate of introduced egg cell destruction (i.e. left open with no eggs remaining) during the first 3 hours of the experiment was tested using a Cox's Proportional Hazards survival analysis. The maximal model included treatment, competition status and an interaction term as explanatory variables. The first of the checks (each 30 minutes) in which an introduced egg cell was identified as destroyed was regarded as the event time, and cells not destroyed within 3 hours were included as censored data (i.e. still informative because the cells were not destroyed before this time). The model was simplified by removing non-significant terms in significance order.

In order to evaluate the effect of treatment and competition status on introduced egg cell fate at 20 hours, a generalised linear mixed model was used with a binomial error distribution and logit link function. Whether or not the egg cell was destroyed within 20 hours of introduction was used as a binary response variable. The maximal model

included treatment, competition status and an interaction term as fixed factors, and included host colony as a random factor, with random slopes for treatment and competition status.

Within introduced egg cells which had not been destroyed within 20 hours, the effect of treatment and competition status on the proportion of eggs eaten was analysed using a linear mixed model. The arcsine transformed proportion of eggs remaining was modelled as the response variable (Crawley 2007). The maximal model included treatment, competition status (pre- or post-competition point) and an interaction term as fixed factors, and included host colony as a random factor, with random slopes for treatment and for competition status (as a single colony could have measures for each level of both of these variables).

For each mixed model, model simplification was performed by removing terms, starting with random terms (except the term for random intercepts per host colony), and then the fixed interaction term. Models were compared using likelihood ratio tests, and where models differed significantly, the model with the lowest AIC value was accepted. Where models did not differ significantly, the model with the fewest terms was accepted. P-values were obtained for each term based on the likelihood ratio chi-squared value when the term was removed from the model. All statistics were performed using R version 2.15.2 (R Development Core Team 2012) and using the lme4 and survival packages (plus all prerequisite packages).

Results

Competition status

In two colonies, the competition point occurred at, or before, the start of the experiment. In one colony, the competition point had not occurred by the end of the experiment. In the remaining seven colonies, the competition point (CP) occurred during the experiment (mean ± SD day: 8 ± 4 of the 17 experimental days). The number egg introductions in each of the four introduction types were as follows: natal, pre-CP = 19; non-natal, pre-CP = 22; natal, post-CP = 40; non-natal, post-CP = 38.

Colony response and cell fate

The rate at which introduced egg cells were destroyed in the first three hours after introduction was not significantly affected by treatment (Cox PH survival analysis, z = -

0.29, d.f. = 2, $p = 0.77$), by competition status ($z = -1.03$, d.f. = 1, $p = 0.31$; fig. 3.1), or by an interaction between treatment and host colony competition status ($z = -0.49$, d.f. = 3, $p = 0.63$).

The destruction of introduced egg cells by 20 hours was not significantly affected by treatment (GLMM, $\chi^2 = 0.26$, $n = 10$ colonies, $p = 0.613$), host colony competition status ($\chi^2 = 0.06$, $p = 0.812$) or an interaction ($\chi^2 = 1.23$, $p = 0.270$; fig. 3.2).

There was no significant effect of either treatment (LMM, $\chi^2 = 1.06$, $n = 10$ colonies, $p = 0.304$), host colony competition status ($\chi^2 = 0.07$, $p = 0.786$), or an interaction ($\chi^2 = 0.15$, $p = 1$) on the proportion of eggs remaining in intact introduced egg cells at 20 hours. Indeed, the mean proportions of eggs remaining for each introduction type, when averaged across all introductions from all colonies (including destroyed egg cells), were highly similar (means ± SE: natal, pre-CP = 0.24 ± 0.09; non-natal, pre-CP = 0.15 ± 0.07; natal, post-CP = 0.28 ± 0.06; non-natal, post-CP = 0.27 ± 0.06; fig. 3.3).

Video observations

Among the 15 filmed introduced egg cells, two had no eggs present at the start of filming (presumably because egg-eating had already occurred in the <30 minutes between introduction and the start of filming). In the remaining 13 filmed introductions, one was an egg cell which had all eggs remaining after 20 hours (based on removal of the cell at this time). As expected, no egg-eating was recorded for eggs in this cell. Among the other 12 filmed introduced egg cells with eggs present at the start of filming, egg-eating of eggs within the introduced egg cells was observed in all cases. Specifically, egg-eating by both workers and the queen was observed in 2 cases, egg-eating by the workers alone was observed in a further 8 cases, and egg-eating by the queen alone was observed in a further 2 cases.

Worker egg-laying into the introduced egg cell was observed in one of the 15 filmed introductions, and queen egg-laying into the introduced egg cell was observed in one further filmed introduction. Because egg-laying was partially obscured, and multiple eggs may have been laid in any one session, it was not possible to determine the number of eggs laid during filming. In these two cases, no eggs remained in the cells at 20 hours. If egg-laying occurred in other cases, it is possible that these eggs could have remained in introduced egg cells until 20 hours, and thus been erroneously counted as introduced eggs evading policing. Nonetheless, the low frequency of egg-laying was unlikely to have affected our conclusions (see *Materials & Methods*).

In all 15 filmed introductions, workers and/or the queen was observed opening the cell to expose eggs, suggesting that the opportunity to access and eat eggs was always present during the experimental introductions.

Discussion

To test whether eggs laid by non-natal workers were less likely to be policed by host colonies than eggs laid by natal workers and the effect of colony stage on policing, we introduced eggs from natal and non-natal workers into pre- and post-competition point *B. terrestris* colonies. We observed varying levels of egg-eating but found no significant difference between eggs laid by natal and non-natal workers in the initial rate of egg cell destruction, the frequency of egg cells destroyed after 20 hours, or the proportion of eggs remaining in intact cells after 20 hours. In addition, whether the receiving colony was pre- or post-competition point had no significant effect on any of these measures of policing.

It is possible that the degree of egg-eating in this study was a product of the artificial way the introduced egg cells were constructed. However, since not all eggs were eaten in all introduced egg cells, any real difference in response to natal and non-natal eggs should have been reflected. Furthermore, the structure of introduced egg cells did not inhibit the ability of individuals to open them, since all filmed introduced egg cells were opened, even if all eggs remained at the end of 20 hours. This suggests that individuals were able to contact eggs directly, and so make decisions based on the eggs themselves, in most or all cases. The reasons for some eggs being left may be due to variations in their surface chemistry, perhaps due to the age of eggs, which was not recorded in this experiment, but which should not have systematically varied across treatments. As in Zanette et al. (2012), we found a high degree of variation in the rates of egg-eating across colonies (e.g. colony 1A vs. colony T4; fig. 3.2 & 3.3). Also in agreement with Zanette et al. (2012), we showed that both queens and workers engaged in egg-eating.

It was already known that bumble bee workers can discriminate between queen and worker eggs (Zanette et al. 2012), as in honeybees (Nanork et al. 2007). However, unlike honeybees (Pirk et al. 2007) or ants (Helantera and Sundstrom 2007; Meunier et al. 2010; Chernenko et al. 2011), our results suggest that bumble bee workers are unable to distinguish between eggs-laid by natal and non-natal workers. Although it is worth noting that the honeybee study (Pirk et al. 2007) compared worker-laid female eggs (produced by a thelytokous strain of honey bee workers), whereas this study

instead compared worker-laid male eggs, which are much more common across eusocial Hymenoptera (Heimpel and de Boer 2008). Nonetheless, the current finding that non-natal worker-laid eggs are not discriminated in bumble bees is consistent with the previous finding that adult males produced from non-natal workers can be observed in wild colonies (Takahashi et al. 2010). Unlike other studies demonstrating the evasion of policing by worker-laid eggs (Oldroyd and Ratnieks 2000; Martin et al. 2002), the failure of individuals in our study to discriminate non-natal worker-laid eggs presumably occurred without any active attempt by the laying workers to disguise eggs, because non-natal (as well as natal) eggs were taken from isolated workers and not from workers which had chosen to infiltrate foreign colonies. Conceivably, drifting workers in nature could disguise eggs by either altering their physiology to produce eggs with a different signature (i.e. chemical mimicry; Lenoir et al. 2001) or by laying their eggs into queen-produced egg cells, which may confer a survival advantage to the eggs (Zanette et al. 2012). However, it seems unlikely that this would confer an advantage solely to non-natal workers, because any such mechanism should presumably also be used by reproductive natal workers (assuming natal workers would also prefer their own eggs not to be eaten). From the perspective of the host colony, the fact that a high proportion of all worker-laid eggs were policed in our study may suggest that specific discrimination of eggs laid by non-natal workers is unnecessary, for example if all worker-laid eggs, regardless of worker origin, are eaten upon being detected as such.

As the frequency or rate of egg-eating did not differ according to whether or not the host colony had passed the competition point, this suggests that individuals in the colony do not alter the response to worker-laid eggs as the stage of colony development changes. Thus, in contrast with our prediction, we showed that worker-laid eggs in pre-competition point colonies are not more likely to escape policing. Mechanistically, this means that the high levels of policing normally observed in colonies after the competition point are not caused by changes in colony-wide aggression, or by differences in an individual's own internal ovary activation, but rather an inherent and fixed response to the presence of worker-laid eggs, which are abundantly present at this colony phase. This is of significance for workers choosing to drift to another colony, because it suggests that the life history stage of the host colony is, other things being equal, not important for the survival of their eggs. In practice, a drifter worker might still be best parasitising a colony before the competition point, since this should represent the best opportunity to produce a son with a high chance of mating (Lopez-Vaamonde et al. 2004); but this assumes the worker has already

activated her ovaries, which may be a requirement for socially parasitic drifting in bumble bee workers (Yagound et al. 2012). However, it is also possible that responses to drifter workers themselves (as opposed to their eggs) differ according to the competition status of the host colony, as non-natal workers can be aggressed (Lopez-Vaamonde et al. 2004), and within-colony aggression in general increases after the competition point (Duchateau and Velthuis 1988).

The results also suggest that, since the threat of policing appears to be constant before and after the competition point, workers do not refrain from egg-laying earlier in colony development due to a greater threat of policing at this time. The results are therefore in contrast with the prediction of Ohtsuki & Tsuji (Ohtsuki and Tsuji 2009), who proposed that policing in eusocial insects evolved to prevent worker egg-laying at the early stages of colony development. Instead, the results highlight the importance of self-restraint in keeping workers from laying before the competition point. This evidence that policing is not a determinant of worker restraint in early colony stages is consistent with the finding in *Camponotus* ants, where there is no policing of worker-laid eggs at this stage (Moore and Liebig 2010).

Because the study revealed no differences in egg cell destruction or egg-eating across all four treatment groups, it strongly implies that a single worker-egg policing mechanism can act to serve two functions: 1) the destruction of eggs laid by rival natal workers; and 2) the destruction of eggs laid by non-natal drifter workers. This is evidence for a role of social parasitism by non-natal workers in the evolution of policing, because the policing response is effective in removing the majority of eggs laid by non-natal workers (approximately 75% eaten within 20 hours), even at times when natal workers do not lay eggs, i.e. before the competition point. However, an additional explanation is that worker-laid egg eating arose to police eggs laid by natal workers but is nonetheless mechanistically responsive throughout colony development (because it is not costly to be responsive at all times). This interpretation is likely, since rates of drifting in bumble bee colonies are fairly low (e.g. ca 1% of adult workers in wild colonies of *B. deuteronymus*; Takahashi et al. 2010), and so may not have provided sufficient selection for policing to evolve. Nonetheless, rates of social parasitism may have been higher in the past (Zanette et al. 2012) and both roles of policing may together have been responsible for the evolution of worker-laid egg policing.

In conclusion, we provide evidence that individuals in bumble bee colonies do not discriminate between eggs laid by natal and non-natal workers. Furthermore, we show

that worker-laid eggs are still eaten before natal workers begin to lay eggs. This may indicate that defence against drifter workers has provided selection towards the same policing mechanism that destroys the eggs of natal workers (since the two cannot be distinguished). A fuller understanding of the potential role of drifters in the origin of policing in *Bombus* will require a comparative analysis, in which more data about the extent of drifting is collect for different *Bombus* species and compared using a phylogeny (e.g. Cameron et al. 2007). It would also be interesting for future studies to determine the identity and impact of the egg cells into which drifter workers and natal workers naturally lay eggs, since this may confer a survival advantage to these eggs and add an extra dimension to the data presented here.

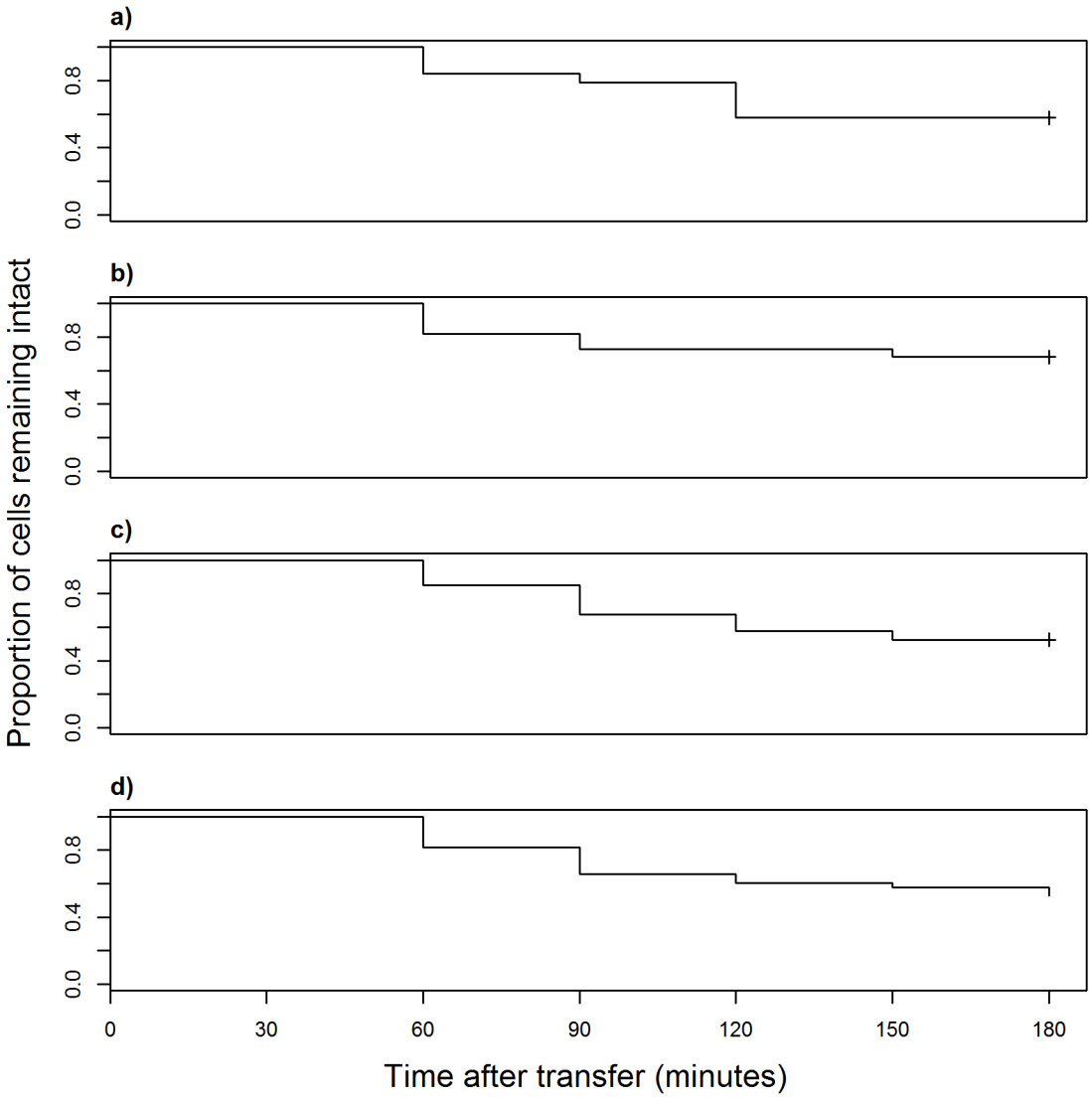

Figure 3.1 The proportion of artificial egg cells introduced to *Bombus terrestris* colonies (n = 10 colonies) intact at 30, 60, 90, 120, 150 and 180 minutes after introduction. The egg cells contained eggs by workers isolated from either the host colony (natal) or another colony (non-natal). The host colonies were either before or after the competition point (CP), a point after which egg-laying by workers in the natal colony occurs. a) = natal, pre-CP (n = 19); b) = non-natal, pre-CP (n = 22); c) = natal, post-CP (n = 40); d) = non-natal, post-CP (n = 38).

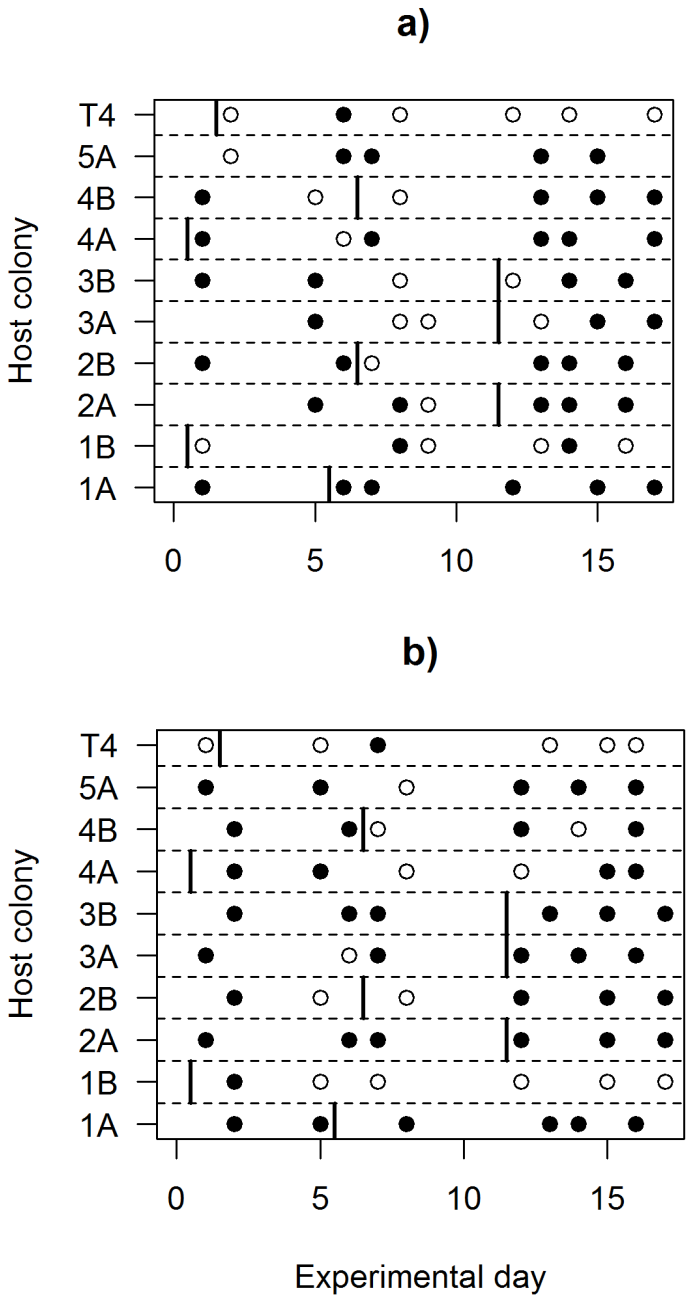

Figure 3.2 The fate of egg cells 20 hours after introducing to host colonies of *Bombus terrestris*, shown for: a) cells containing eggs from natal workers, and b) cells containing eggs from non-natal workers. Each host colony was given 11-12 introduced egg cells over the course of the experiment (x-axis), each depicted by a separate circle. Circles are filled depending on cell fate: filled circles = destroyed cell (cell opened with all eggs removed); open circles = cell not destroyed. Thick vertical lines indicate the date of the competition point for each colony.

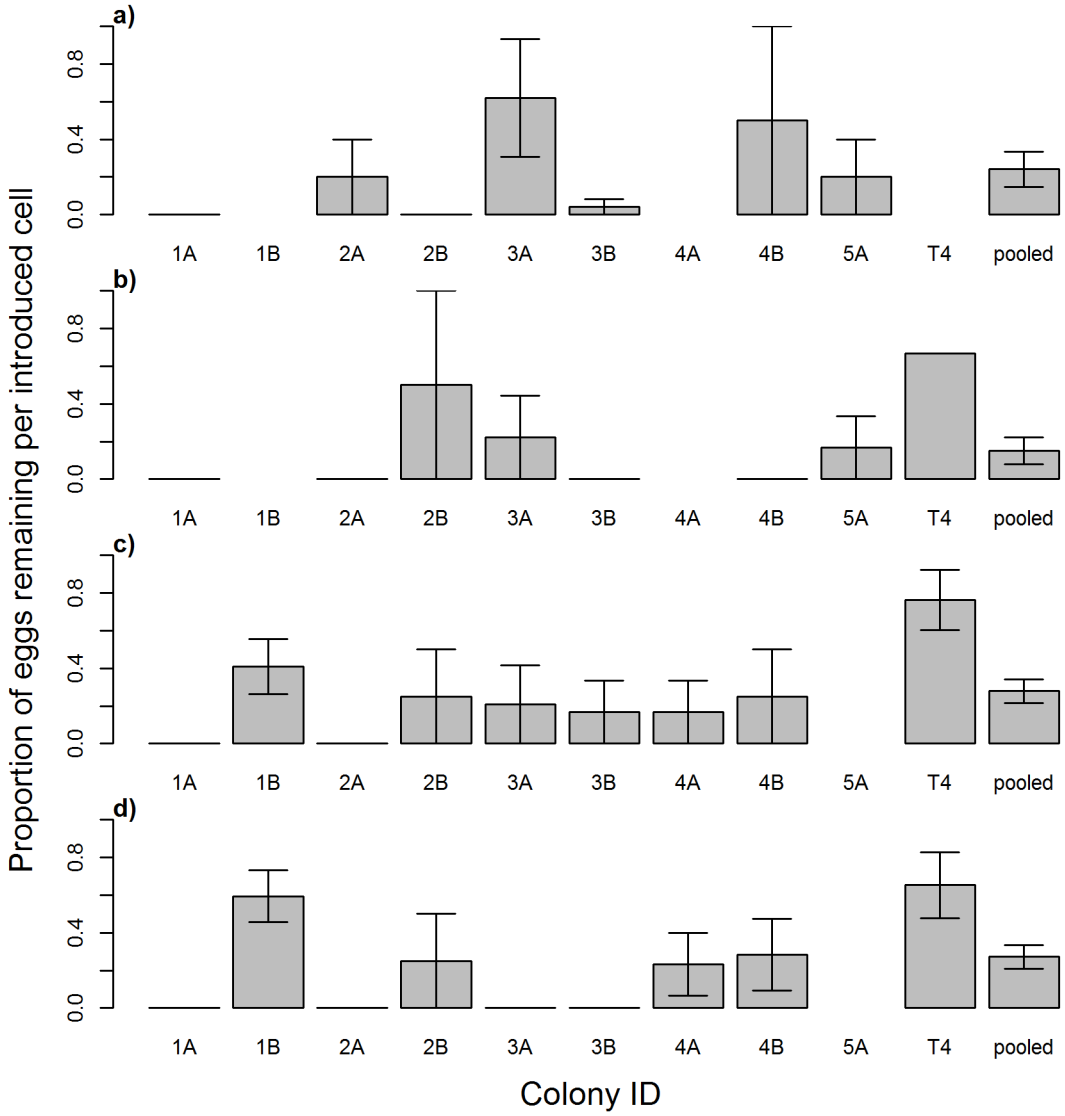

Figure 3.3 The mean proportions of eggs surviving in introduced egg cells after 20 hours, separated by host colony and introduction type. a) natal, pre-CP (n = 19); b) non-natal, pre-CP (n = 22); c) natal, post-CP (n = 40); d) non-natal, post-CP (n = 38). 0–6 introductions per introduction type, per host colony. Means pooled across colonies are also shown at far right. Horizontal lines with no error bars indicate no eggs remained for all introductions; blank plots indicate no introductions. Error bars, SEs.

Chapter 4

Effects of temperature on colony life history in the bumble bee Bombus terrestris

Abstract

Pollinating insects are of huge ecological and commercial importance, and understanding how they respond to climate change is a research priority. Despite this, few studies have investigated the life history responses of pollinators to experimentally controlled changes in temperature, which should be especially informative for species with complex life histories, such as eusocial insect colonies. Over two laboratory experiments, we reared 44 developing colonies of the bumble bee *Bombus terrestris* at either 20^{o}C or 25^{o}C, and measured a number of life history traits including: colony longevity, queen longevity, worker longevity, production of workers, production of sexuals and growth schedule, as well as thermoregulation behaviours. We found that temperature only had marginal or non-significant effects on colony longevity, queen longevity and worker longevity, and propose that the lack of an effect on individual longevity may be explained by observed thermoregulatory behaviours acting to buffer external effects. No response was found in the timing of male production or peak colony size, suggesting that colonies may be vulnerable to a mismatch in timing with food plants. However, higher temperature increased colony size and the production of new queens, demonstrating that temperature affects colony-level productivity. In a third experiment, we tested the hypothesis that higher temperatures make colony foundation in the absence of queen diapause more likely by rearing non-diapaused mated queens at 15^{o}C or 20^{o}C. However, very few non-diapaused queens laid eggs in either treatment. The study fails to find evidence for, but does not completely refute, the recently suggested hypothesis that winter-activity of bumble bees in Britain is explained by climate warming. More generally, it adds much needed data on the basic responses of bumble bee life history to temperature, and should be integrated with further research in order to understand and predict responses of pollinating eusocial insects to climate change.

Introduction

In light of global climate change (Solomon et al. 2007), exploring the role of temperature is an increasingly important component of understanding the life history of any species. Crucially, large scale perturbations in environmental temperature might be fatal in species that fail to adjust their life histories adaptively (Sinervo et al. 2010). This is especially important to consider in organisms which perform vital ecosystem services, such as pollinators (Brown and Paxton 2009; Vanbergen et al. 2013). Over a third of global food production is dependent on pollination (Kjøhl et al. 2011), but many pollinators are currently in decline (Abrol 2012; Vanbergen et al. 2013). Several recent studies have explored the relationship between pollination and climate change by tracking the observed phenologies (annual appearance times) of plants and their pollinators (Gordo and Sanz 2005; Memmott et al. 2007; Bartomeus et al. 2011). These studies modelled the effect of temperature and other climate variables on phenology, and have variously predicted that future climate trends will result in either the disruption (Gordo and Sanz 2005; Memmott et al. 2007) or maintenance (Bartomeus et al. 2011) of current plant-pollinator interactions. One specific concern is that differential phenological responses of plants and pollinators to temperature may result in a 'mismatch in phenology', resulting in declines in plants, pollinators or both (Stenseth and Mysterud 2002; Visser and Both 2005; Miller-Rushing et al. 2010). Whilst these models are important, they do not address the underlying mechanisms controlling life history responses of individual pollinator species, and so may misrepresent responses of certain organisms to key variables such as temperature (Memmott et al. 2007; Visser et al. 2010). Although the responses of insect life history to temperature has been studied in a number of cases (e.g. Calabi and Porter 1989; Sheehy 2002; Isitan et al. 2010), data are lacking on key pollinator species (but see Bosch et al. 2000; Karlsson and Wiklund 2005). In particular, many of the most important pollinating insects are eusocial, e.g. honeybees, bumble bees and stingless bees (O'Toole 1993; Chapman and Bourke 2001), and yet to our knowledge, no published data exist on the direct effects of temperature on bee colony life history traits such as colony longevity and colony productivity. In eusocial systems, groups of many individuals can live synergistically in colonies, and so the colony itself possesses life history characteristics such as growth, reproduction and decline (Wilson 1985; Bourke 2011; Holland et al. 2013). This means that environmental factors, such as temperature, could have effects

on colonies which are different from those usually expected in individual organisms. For example, a temperature change which elicits a reduction in the longevity of an individual within a colony may not reduce the longevity of the colony as a whole.

The bumble bee *Bombus terrestris* is an ideal candidate species for investigating life history in a pollinating eusocial insect because it possesses an annual colony cycle, meaning large components of its life history, both at an individual and colony level, can be studied over a short time frame. Moreover, the species is a key pollinator of both commercial crops and wild flowers (Goulson 2010), and so globally important in its own right. Typically, colonies are established in spring by lone queens, and initially consist of workers (mostly-sterile females), developing brood and the foundress queen. In summer, the colony also produces sexually capable individuals (sexuals) in the form of new queens and males, which disperse and mate to found new colonies. All workers and sexuals are generally the offspring of the queen (although a small number of males can be produced by workers). The colony begins to decline in the late summer, with the colony queen and all workers eventually dying, usually by October (Prŷs-Jones and Corbet 2011). The newly-mated queens enter diapause and thereby hibernate through the winter, emerging the following spring to found the next generation of colonies. However, variations in life history exist in populations residing in different climactic conditions. For example, *B. terrestris* populations in the Mediterranean region exhibit phenologies different from those found in northern European populations, and queens in this region aestivate during the warm dry summer rather than hibernating during the winter (Gurel et al. 2008; Rasmont et al. 2008). Populations in the Mediterranean region, New Zealand and Tasmania also appear to have two colony cycles per year (bivoltinism; Donovan and Wier 1978; Buttermore 1997; Rasmont et al. 2005).

One example of a potential shift in life history that has been noted in recent years is the presence of winter active *B. terrestris audax* in Britian. Sightings of both queens and workers between October and January (i.e. outside the expected season) have been frequently reported in the last 10-20 years but such sightings have not been reported historically (Robertson 1991; Edwards 2006; Farmer 2006; Goulson 2010; Stelzer et al. 2010; Bees Wasps and Ants Recording Society (BWARS), personal communication). The reasons for this phenomenon are not clear, although it is possible that recent changes in seasonal temperature have played a role (Stelzer et al. 2010), adding to the case that climate change may already be affecting bumble bees. In general, life history changes of an organism in response to temperature might be the result of phenotypic plasticity, or of population genetic changes (Nylin and Gotthard 1998). In both cases,

these changes may or may not be adaptive, because a species could fail to respond appropriately in the short or long term (Nylin and Gotthard 1998). In the case of *B. terrestris audax*, there are two, non-mutually exclusive mechanisms that might explain the occurrence of winter-active bees. Firstly, increasing temperature during colony development (e.g. summer temperature) could have increased the longevity of colonies, meaning active workers and queens persist into the winter. Secondly, increasing autumn temperature may have caused queens to found a winter generation of colonies without diapause. Not enough is known about the relationship between temperature and the life history of *B. terrestris* to be able to evaluate these possibilities. In other animals, lower body temperatures may increase longevity by, for example, reducing oxidative damage (Conti 2008). Indeed, lower ambient temperatures have been found to increase longevity in a number of insects (Calabi and Porter 1989; Sheehy 2002; Isitan et al. 2010), and so the same might be expected in bumble bees. However, in mature bumble bee colonies, the nest environment is maintained at a roughly constant temperature (Heinrich 1979; Vogt 1986; Weidenmuller et al. 2002) (i.e. colony-level thermoregulation), meaning individuals in the nest may not respond in the same way as solitary animals. Furthermore, colonies with longer-lived workers might not themselves persist for longer, as the timing of worker production might not remain constant. The influence of temperature on producing a second annual generation is plausible because higher temperatures have been associated with increasing the number of generations per year in other insects (e.g. Jonsson et al. 2009; Robinet and Roques 2010). Furthermore, ovary activation in post-diapaused queens of *B. terrestris* and *B. impatiens* have been shown to increase in response to higher abdominal or ambient temperatures (Vogt et al. 1998; Amin et al. 2008), demonstrating that temperature can influence the preparedness for colony founding.

In this study we investigated the role of temperature in colony life history, focussing particularly on the factors which may be causing winter activity of *Bombus terrestris* in Britain, with the expectation that our results would also lead to more general implications for the conservation of pollinators and social insect biology. We used laboratory manipulations to alter the ambient temperature of *Bombus terrestris* colonies and queens. We used colony and individual-level life history observations to measure the relationship between temperature and life history, with emphasis on possible explanations for winter activity. Specifically, we tested the hypotheses: 1) that increasing the ambient temperature of colonies increases colony longevity; and 2) that

increasing the ambient temperature of newly-mated queens increases the chance of colony foundation without diapause (i.e. a second annual generation).

Materials & Methods

In order to investigate the effects of ambient temperature on *Bombus terrestris audax* life history, three laboratory experiments were conducted. Experiment 1 was concerned with how changing ambient temperature might affect colony longevity, colony queen longevity, the production of sexuals, and workers' thermoregulatory behaviour. In order to increase sample size, Experiment 2 repeated Experiment 1, but also included an analysis of the effect of temperature on the longevity and production of workers. Due to the similarity in the techniques between these two experiments, and to facilitate the explanation and interpretation of methods and results, they are largely considered together. For Experiments 1 and 2, the ambient temperature treatments used were 20^{o}C and 25^{o}C. Since constant temperatures were used, late summer temperatures (southern England mean daily maximum temperatures 1981-2010; July = 21.6^{o}C, August = 21.4^{o}C; Met Office 2013) were chosen so that they would simulate the approximate maximum temperatures mature colonies in southern Britain would experience, in average years and warmer years respectively. Finally, in order to explore whether high ambient temperatures induce bivoltinism, Experiment 3 tested the effect of ambient temperature on the incidence of oviposition (egg-laying) in mated, pre-diapause *Bombus terrestris terrestris* queens. Using this central European subspecies in Experiment 3 allowed for consistency and comparability with a previous study demonstrating pre-diapause oviposition (Beekman et al. 1999). The temperature treatments selected for Experiment 3, 15^{o}C and 20^{o}C, were lower than for Experiments 1 and 2, since this lower temperature range would be more realistic for the usual time of queen mating in Britain (southern England mean daily maximum temperatures 1981-2010; August = 21.4^{o}C; September = 18.5^{o}C, October = 14.4 ^{o}C; Met Office 2013).

Experiments 1 and 2 – Colony longevity, individual longevity, colony productivity and thermoregulation

Colony culture and treatments

Mature colonies of *Bombus terrestris audax* were obtained from a commercial supplier (Biobest, Westerlo, Belgium). For both experiments, all colonies were queenright (i.e.

contained a colony queen) but, at the start of the experiment, had not yet produced any adult sexuals. Colonies at this stage were selected in order to allow effects on the production of sexuals to be observed. For Experiment 1, we used 24 colonies received on 4 August 2011 and then acclimatised in a controlled-environment (CE) room for 5 days at 28^{o}C, followed by 2 days at 22.5^{o}C before the start of the experiment. For Experiment 2, we used 20 colonies obtained on 3 February 2012. The experiments started on 11 August 2011 and 4 February 2012, respectively. On the first day of each experiment, or one day before, the number of workers in each colony was counted (Experiment 1, mean (range) = 62 (41-94), n = 24 colonies; Experiment 2, mean (range) = 62 (34-89) n = 20 colonies). Each colony (including workers, queen and brood) was then transferred to a wooden nest box (300 × 200 × 170 mm) with a clear plastic lid. Each colony was supplied with sugar syrup nectar substitute (Biobest) via a wick through a hole in the bottom of the nest box connected to a syrup container underneath. Each colony was also given approximately 9 g of dried pollen (Biobest) every two days. This amount of pollen was selected because it was generally consumed within 1-2 days; ad libitum feeding was avoided because it might have allowed the lower temperature colonies to equalise energy levels and negate any differences with the higher temperature colonies. As colonies declined in worker number as the experiment proceeded, the amount of pollen was reduced whenever pollen remained after 2 days in at least one colony, but was always kept equal across all colonies. From the start of each experiment, colonies were kept in a CE room set at 20^{o}C and 60%RH (Experiment 1) or 50%RH (Experiment 2). The humidity was reduced for Experiment 2 in order to reduce the accumulation of moisture in nest boxes which occurred in some colonies in Experiment 1. The CE room was kept in constant darkness for both experiments, except when colonies were being observed or manipulated, at which times colonies were illuminated using red light (which is invisible to bumble bees).

In each experiment, colonies were randomly divided into two groups (Experiment 1, 12 colonies per treatment; Experiment 2, 10 colonies per treatment), exposed to either a 20^{o}C or 25^{o}C treatment. In both Experiment 1 and 2, the initial colony sizes were not significantly different across treatments (Experiment 1, t-test, t_{22} = 0.16, p = 0.873; Experiment 2, t-test, t_{18} = 0.17, p = 0.871). Colonies in the 20^{o}C treatment were placed on bench tops within the 20^{o}C controlled-environment room. Colonies in the 25^{o}C treatment were placed on bench-tops within the same room but were also provided with electric heat mats (Repti-zoo, Fenggang Town, China) that were inserted

underneath the nests between the nest box and the syrup container. In order to prevent heat mats from interrupting access to the syrup wicks, the heat mats were positioned to cover approximately two thirds of underside of the nest box, always underneath the brood comb. Heat mats were temperature-controlled using thermostats (Repti-zoo), with the thermostat probes attached to the surface of the heat mats themselves. Thermostats were set to a level that heated empty nest boxes to 25^{o}C (as indicated by preliminary testing). Probes were not placed inside nest boxes as this would have caused temperature to be adjusted in response to the level of colony thermoregulation and would therefore have not provided a fixed temperature treatment. In-nest temperatures were monitored throughout both experiments (see *Colony Thermoregulation*).

Colony and individual longevity

In Experiment 1, colonies were monitored approximately daily (mode (range) = 1 (1-3) days between observations) for colony death, i.e. when the number of living adult workers first reached zero. Colony longevity was defined as the time in days between the start of each experiment and the date on which colony death was reached. In one colony, two adult workers remained alive on the date the colony was terminated; for this colony, four days were added to its colony longevity, because four days was the average time taken, in colonies at the end of their lives, for adult worker number to fall from two to zero. In Experiment 2, colonies were terminated when all workers analysed for longevity (see below) had died. Before this time, colonies were monitored for colony death in the same way as Experiment 1. In addition, three colonies ran out of syrup on or after day 58, and so were excluded from all analyses after this time (see *Statistical Analyses*).

In Experiment 1 and 2, colonies were monitored approximately every 2 days (mode (range) = 2 (1-3)) for queen (foundress) death. Queens were differentiated from new queens by identifying wear in wings and hairs which is usually present in foundress queens but not new queens (Prŷs-Jones and Corbet 2011). Queen longevity was defined as the time in days between the start of the experiment and queen death.

In Experiment 2, worker longevity (time in days between worker eclosion and date of death) was also measured by marking a subset of workers with individually-numbered tags. Beginning on day 1 of the experiment, all colonies were checked daily for the presence of callow workers (newly-emerged adult workers), which were readily

identified due to the lighter colour of their hairs. As callow workers are often inactive and hidden from view (personal observation), it is likely that some evaded detection, but we assumed this to be consistent across all colonies. For each colony, all identified callow workers were marked daily for seven days following the eclosion of the first recorded callow from that colony or, if ten workers had not been marked by that time, until at least ten workers were marked. To identify the effect of emergence time on longevity, a second contingent of callow workers was marked, according to the same protocol, starting with the first recorded callow workers eclosing after day 20. Callow workers were marked using numbered coloured tags (Buzzy Bee Shop Ltd, Leeds, UK) attached to their thoraces with glue. Approximately every day (mode (range) = 1 (1-3) days between observations), colonies were checked for marked workers, and each was recorded as either seen, not seen or dead. If a detached disc was found in the nest, the individual to which that disc had belonged was excluded from the analysis. As all marked workers were not seen every day, the date of death was estimated for each worker as the mean of the date last seen alive and the date first seen dead (mean ± SD number of days between these two dates = 4.3 ± 7.3 days). Worker longevity was defined as the time in days between the date of worker eclosion and the above estimate of worker death.

Colony productivity

In Experiments 1 and 2, the number of adult workers in each colony was censused using a mechanical tally counter approximately once per week (Experiment 1, mean (range) = 6.9 (5-9); Experiment 2, mean (range) = 7.8 (5-13) days between observations). In colonies with fewer than 20 workers, the exact worker number was counted, and in colonies with more than 20 workers, worker number was estimated to the nearest 5. In Experiment 2 only, the production of workers was also estimated by recording daily all observed callow workers from day 1 to day 49. The recording of callow workers ceased at this time because almost all callow adults were males by this point. As stated above (see *Colony and individual longevity*), callow workers may have sometimes evaded detection and so counts of callow workers may have been underestimates (but the magnitude of underestimation should not have systematically varied across treatments). In Experiment 1 only, each colony was checked for sexuals approximately every 2 days (mode (range) = 2 (1-3) days), and all sexual individuals detected in this way were counted and removed. Removal of sexuals simulated the situation in wild nests, in which the sexuals usually depart within a few days of eclosion

(Alford 1975). The first day on which males were detected per colony was compared between treatments. In Experiment 2, sexuals were not removed but the number of new queens produced per colony was recorded. The number of males present in each colony was also assessed at least once per 10 day period. The first week in which males were detected per colony was compared between treatments. The timing of new queen production was not compared since only a small number of colonies produced new queens.

Colony thermoregulation

In both Experiments 1 and 2, internal nest temperatures were measured to assess the level of thermoregulation between the treatments. Measurements were conducted using a datalogger (EL-USB-2; Lascar Electronics, Salisbury, UK) enclosed by a small plastic bag and placed inside the nest box contacting the brood comb and one wall of the box. Two dataloggers were used for measurements simultaneously in different colonies. Over a series of periods of approximately 45 h each, one datalogger was placed in a 20^{o}C treatment colony and the other in a 25^{o}C treatment colony. After each period, dataloggers were switched to another pair of colonies; the order of colonies was determined randomly and each datalogger was alternated between treatments. In Experiment 1, this procedure was used to measure the internal temperature of each colony once during the first 5 weeks of the experiment. In Experiment 2, to increase the number of measurements, the internal temperature of each colony was instead measured a mean (range) of 1.8 (1-3) times during the first 7 weeks of the experiment. The role of thermoregulatory behaviour in maintaining nest temperature was also investigated. This was done by recording (1) wing fanning in Experiment 1 only, which acts to cool nests (Vogt 1986; Weidenmuller et al. 2002), and (2) wax canopy building in Experiments 1 and 2, which acts to insulate nests from heat loss (Heinrich 1979). In Experiment 1 only, during each of the first three weeks, each colony was digitally filmed (Handycam; Sony, Tokyo, Japan) for two hours (6 hours in total per colony), with the cameras focused on the centre of the nest to allow the observation of fanning behaviour. All filming took place between 09:00 and 20:00 and was conducted in a randomised order each week, with three colonies being filmed at a time. Each two-hour video was later viewed blindly with respect to treatment, with activity being recorded from a field of view of standard size (150 x 150 mm) containing workers and brood comb. Each incidence of fanning behaviour lasting over 10 s was recorded and the nest fanning rate (number of incidences per minute) for each video was calculated. In

order to calculate a fanning rate per individual, the average number of workers across the duration of each video was estimated by counting the number of workers in the selected field of view at 10-minute intervals and then calculating the mean of these values. The nest fanning rate was then divided by the average number of workers for each video, in order to produce an individual fanning rate (number of incidences per minute per individual). In Experiment 1 and 2, wax canopy building behaviour was measured by estimating (to the nearest 5%) the percentage cover of wax canopy over the brood comb of each colony on day 27 of Experiment 1, and on days 15 and 27 of Experiment 2.

Statistical analyses

In Experiment 1, colony longevity was compared across the two treatments using a t-test. In Experiment 2, these data were analysed using a survival analysis (Crawley 2007). Colonies that either (a) contained living workers at the end of the experiment (n = 3 colonies) or (b) provided counts only up to the point where they depleted all their syrup (n = 3 colonies), were included in this analysis as right-censored data (i.e. colonies for which death would occur after, but not before, their respective censoring dates).. A Cox's proportional hazards (Cox's PH) model was used with colony death as the event time variable and treatment as the predictor variable. A second Cox's PH model was used with the first census date at which a colony declined to 10 workers as the event time variable, because most colonies had reached this state by the end of the experiment (n = 17).

In Experiment 1, the longevity of queens was compared across the two treatments using a t-test. In Experiment 2, the longevity was compared using a Cox's PH model with queen death as the event time variable and treatment as the predictor variable. This model included two right-censored values due to one queen, among the colonies running out of syrup, which had not died by this time, and a further queen which had not died by the end of the experiment.

In Experiment 2, the effect of temperature on worker longevity was analysed using a linear mixed model (LMM). Worker longevity was included as the response variable, with treatment and contingent (1 or 2 depending on marking date, see *Colony and individual longevity* above) as fixed factors, and colony ID as a random factor (with random intercepts and slopes over time, per colony) in the maximal model. All marked workers from which the tag was lost in the nest, or whose death occurred after syrup

depletion in a colony, were not included in the analysis as their time of death could not be known (n = 126). This left 160 workers included in the analysis (20^{o}C treatment: n = 72 workers from 10 colonies; 25^{o}C treatment: n = 88 workers from 10 colonies).

In Experiments 1 and 2, to analyse the effect of temperature on the total number of adult workers per colony over time, generalised linear mixed models (GLMMs) were used with a Poisson error distribution and a log link function. The count of workers on each census date (see *Colony Productivity*) was used as the response variable. Temperature treatment, measurement day and an interaction were included as fixed variables, and colony ID as a random variable (with random intercepts and slopes over time, per colony) in the maximal model. Where required for the normality of errors, quadratic terms for measurement day were included as fixed terms in addition to linear terms (as might be expected in growth models). As colony size increased initially and then declined in both experiments and both treatments, separate models were used for growth and decline phases for each experiment. The separation between these two phases was taken to be the mean time at which the number of workers per colony was largest, taken separately for each experiment (Experiment 1: day 31; Experiment 2: day 33). All measurements taken before (and including) this time were included in the respective growth model, and all measurements taken after this time were included in the respective decline model. In addition, the week on which the peak worker number per colony was reached was compared between treatments using a t-test. In Experiment 2, the number of callow workers produced per colony (in days 1-49; see *Colony Productivity*) was compared between treatments using a t-test.

In Experiments 1 and 2, independent tests were performed for the effects of treatment on numbers of sexuals produced per colony, as follows. The number of new queens produced per colony was compared between treatments using Wilcoxon's rank sum tests with continuity correction. In Experiment 1, the number of males produced per colony was compared using a t-test on the log-transformed data. The first day of male eclosion was compared between treatments using a t-test. In Experiment 2, only data collected before day 58 were included, as this allowed the inclusion of the three colonies running out of syrup on this day (see *Colony culture and treatments*). As the total number of males produced per colony was not known, a comparison was made between the largest number of males counted on any one census date, per colony, for each treatment using a t-test. The first week of male eclosion was compared between treatments using a t-test.

In Experiment 1, the in-nest temperatures recorded by dataloggers were compared between treatments using a two-way ANCOVA. The maximal model included treatment and measurement date (the day each logging period started) as explanatory variables and mean temperature (over each logging period) as the response variable. In Experiment 2, an LMM was used instead as most colonies were measured more than once (see *Colony Thermoregulation*). The maximal model included treatment and measurement date as fixed explanatory variables, colony ID as a random explanatory variable and mean temperature as the response variable.

In Experiment 1, to analyse the effect of temperature on wing fanning behaviour, a GLMM was used with a binomial error structure and a logit link function. Treatment and time were used as fixed explanatory variables, and colony ID as a random variable (random intercepts and slopes over time). Presence/absence of wing fanning was used as the response variable for the statistical analysis, as no wing fanning was observed in a large number of filming sessions. In Experiments 1 and 2, the percentage cover of wax canopy per colony was compared between treatments using Wilcoxon rank sum tests with continuity correction.

For all analyses, relevant tests to confirm normality (Shapiro-Wilk test) and homogeneity of variance (Fisher's F test or Levene's test for homogeneity of variance) were performed, and parametric or non-parametric tests were used accordingly. Where data were non-normal, $\log_{10}$-transformed data were used where the distribution of these transformed data was normal. Welch's t-tests were used for data not conforming to homogeneity of variance. All tests were two-tailed. Model simplification in LMMs and GLMMs was performed by removing terms from a maximal model. Models were compared using likelihood ratio tests, and where models differed significantly, the model with the lowest Akaike's Information Criterion (AIC) value was accepted (Crawley 2007). Where models did not differ significantly, the model with the fewest terms was accepted. P-values were obtained for each term based on the likelihood ratio chi-squared value when the term was removed from the model. All statistics and figures were produced using R (R Development Core Team 2012) with the lme4 and ggplot2 packages.

Experiment 3 – Oviposition in pre-diapause queens

Queen culture and treatments

Recently mated (but not diapaused) *Bombus terrestris terrestris* queens were obtained from a commercial supplier (Syngenta Bioline Bees, Weert, Netherlands) on 28 June 2012. Each of the queens was randomly assigned to one of three treatment groups: 15^{o}C treatment group (n = 48 queens), 20^{o}C treatment group (n = 48 queens) and diapause treatment group (n = 28 queens). The diapause treatment was included to ensure that the queens were, when exposed to a lower temperature, capable of diapause. Queens in the 15^{o}C treatment group were each placed in a small plastic nesting container (140 x 79 x 60 mm) kept in a CE room set to 15^{o}C and 60%RH in constant darkness. Queens in the 20^{o}C treatment were kept in the same CE room, but their nesting containers were placed on heat mats thermostatically regulated to maintain a temperature of 20^{o}C following the methods in Experiments 1 and 2. All queens in both 15^{o}C and 20^{o}C treatment groups were fed with *ad libitum* sugar syrup (Koppert Biological Systems, Berkel en Rodenrijs, Netherlands) and a dried pollen-syrup mixture replaced every 3 days. Queens in the diapausing treatment group were kept in two large plastic boxes (427 x 312 x 150 mm) each divided into 15 compartments by cardboard dividers, with one queen being placed in each compartment. The boxes were filled to a depth of approximately 90 mm with moist sedge peat (Peregrine Livefoods Ltd, Ongar, UK) in order to allow the queens to burrow, which facilitates diapause (Velthuis 2002). These boxes were kept in an incubator set to 5^{o}C with permanent lighting for the first week. After one week, the temperature was reduced to 4^{o}C and the lighting was permanently switched off in order to further stimulate diapause. Queens in this treatment group were fed ad libitum sugar syrup and pollen-syrup mixture for the first four weeks, after which the pollen and syrup were permanently removed in order to reduce mould growth. This initial feeding was to ensure that queens survived whilst they each transitioned to a diapause physiology (Beekman et al. 1998b). Temperature and humidity levels for all three treatments were tested periodically using data loggers. Loggers were placed either in a box identical to the boxes housing the queens on a bare bench top or on a heat mat next to the queen boxes (first two treatments), or in a box identical to the ones housing the diapausing queens in the incubator (third treatment).

Oviposition and ovary activation

All queens in the 15°C and 20°C treatment groups were checked daily for the presence of eggs. After 1 week, in order to stimulate oviposition, each extant queen in these treatments was given a cocoon containing a dead pupa which had previously been frozen (Kwon et al. 2003; Gurel and Gosterit 2008). The cocoons were obtained from mature *Bombus terrestris terrestris* colonies and randomly assigned to queens. These cocoons were then fixed to a plastic dish with Vaseline before being introduced into each nesting container. After five weeks, in order to further stimulate ovipoisition, a single *Bombus terrestris terrestris* worker was added to each queen (Beekman et al. 1999). For the remainder of the experiment, any dead workers were replaced within 24 h with another live worker. Where eggs were found in boxes containing a worker, the worker was removed to allow confirmation that the queen, and not only the worker, was capable of oviposition. Monitoring ceased after 65 days, which should have left ample time for oviposition, as oviposition in post-diapause queens usually begins before this time, and pre-diapause queens in a previous study oviposited within 42 days (6 weeks)(Beekman et al. 1999).

Diapause

Queens in the diapause treatment were observed throughout the experiment for behavioural signs of diapausing and death. According to previous studies, signs of diapause in bumble bee queens include burrowing behaviour and inactivity (Alford 1969; Goulson 2010), but also survival through a long period without food (Beekman et al. 1998b). These observations were initially performed daily, in order to precisely note the date of any deaths, but the checks were performed with reducing frequency over the course of the experiment, in order to keep disturbance to a minimum, as the death rate amongst these queens was low. After 65 days, all queens were checked for movement to confirm survival and thus infer successful diapausing.

Results

Experiments 1 and 2 – Colony longevity, individual longevity, colony productivity and thermoregulation

Colony and individual longevity

In Experiment 1, there was no significant effect of treatment on colony longevity (t-test, t_{22} = 1.87, p = 0.075, 95% confidence interval of mean increase from 20°C to 25°C = -0.5 – 9.1 days; fig. 4.1), although there was a trend for colony longevity to be greater in the 25°C treatment (means ± SE: 20°C treatment, colony longevity = 57.1 ± 1.7 days, n = 12 colonies; 25°C treatment, colony longevity = 61.4 ± 1.6 days, n = 12 colonies). In Experiment 2, however, colony longevity was significantly higher in the 25°C treatment (means ± SE: 20°C treatment, colony longevity = 89.3 ± 4.0 days, n = 9 colonies; 25°C treatment, colony longevity = 103.2 ± 4.3 days, n = 5 colonies; Cox's PH, z_1 = 2.70, p = 0.007), but there was no effect of treatment on the time taken to decline to 10 workers (Cox's PH, z_1 = 0.25, p = 0.800). In Experiment 1, there was no significant effect of treatment on queen longevity (t-test, t_{22} = 0.43, p = 0.673, 95% confidence interval of mean increase from 20°C to 25°C = -7.0 – 10.7 days; fig. 4.2). In Experiment 2, there was also no significant effect of treatment on queen longevity (Cox's PH, z_1 = 1.48, p = 0.140). In Experiment 2, no effect was found on worker longevity of treatment (LMM, χ^2 =0.24, p = 0.624), though a significant effect was found of contingent (LMM, χ^2 =32.93, p < 0.001), with workers produced in the first contingent living longer than workers produced in the second (means ± SE: contingent 1, worker longevity = 43.9 ± 1.72 days, n = 123 workers from 20 colonies; contingent 2, worker longevity = 24.4 ± 2.6 days, n = 37 workers from 7 colonies; fig. 4.3). No effect was found of a temperature-contingent interaction (LMM, χ^2 =0.62, p = 0.618).

Colony productivity

In Experiment 1, during the growth phase, the number of adult workers per colony (n = 24 colonies) was significantly higher in the 25°C treatment (GLMM, χ^2 = 15.03, p < 0.001) and significantly higher later in the experiment (linear term: GLMM, χ^2 = 38.54, p < 0.001), but there was no effect of a treatment-day interaction (GLMM, χ^2 = 3.40, p = 0.065; fig. 4.4a). In the decline phase, the number of adult workers per colony was again significantly higher in the 25°C treatment (GLMM, χ^2 = 5.00, p = 0.025) and significantly higher later in the experiment (GLMM, linear term: χ^2 = 65.5, p < 0.001, quadratic term: χ^2 = 407.8, p < 0.001), but the number of workers was not significantly affected by a treatment-day (quadratic) interaction (GLMM, χ^2 = 2.83, p = 0.093; fig. 4.4a). In Experiment 2, in the growth phase, the number of workers per colony (n = 20 colonies) was not significantly affected by treatment (GLMM, χ^2 = 2.46, p = 0.117), but

there was a significant positive effect of day (GLMM, χ^2 = 14.60, p < 0.001) and no effect of a treatment-day interaction (GLMM, χ^2 = 2.37, p = 0.124; fig. 4.4b). However, the mean number of workers was consistently higher in the 25°C treatment in this growth phase. In the decline phase, the number of workers was significantly significantly higher in the 25°C treatment (GLMM, χ^2 = 62.30, p < 0.001), significantly higher later in the experiment (GLMM, linear term: χ^2 = 52.36, p < 0.001) and there was a significant treatment-day (quadratic) interaction (GLMM, χ^2 = 67.35, p < 0.001), with the 25°C treatment increasing the speed of the decline in number of workers relative to the 20°C treatment (fig. 4.4b). The week of peak worker number per colony did not differ significantly between treatments in either Experiment 1 (means ± SE: 20°C treatment = 5.3 ± 0.6, n = 12; 25°C treatment = 6.0 ± 0.3, n = 12; Welch's t-test, t_{17} = 1.15, p = 0.267; fig. 4.4a), or Experiment 2 (means ± SE: 20°C treatment = 3.9 ± 0.6, n = 10; 25°C treatment = 4.0 ± 0.4, n = 10; t-test, t_{18} = 0.15, p = 0.884; fig. 4.4b).

In Experiment 2, the total number of callow workers detected per colony was not significantly affected by treatment (t-test, t_{18} = 1.68, p = 0.110), although there was a trend for this number to be higher in the 25°C treatment (means ± SE: 20°C treatment = 37.0 ± 5.5 workers, n = 10; 25°C treatment = 51.1 ± 6.3 workers, n = 10; fig. 4.5).

In Experiment 1, the total number of new queens produced per colony was significantly higher in the 25°C treatment (means ± SE: 20°C treatment mean = 0.8 ± 0.5 new queens, n = 12; 25°C treatment mean = 3.2 ± 1.2 new queens, n = 12; Wilcoxon's signed rank test, W = 102.5, n = 24, p = 0.046; fig. 4.6a). In Experiment 2, this significant relationship was also found (means ± SE: 20°C treatment = 0.1 ± 0.1 new queens, n = 10; 25°C treatment = 6.8 ± 2.9 new queens, n = 10; Wilcoxon's signed rank test, W = 78, n = 20, p = 0.015; fig. 4.6b). In Experiment 1, the total number of males produced per colony was not significantly affected by treatment (means ± SE: 20°C treatment = 15.6 ± 2.8 males, n = 12; 25°C treatment = 17.3 ± 3.9 males, n = 12; t-test, t_{22} = 0.13, p = 0.899; fig. 4.7a). The date of eclosion of the first males by each colony was not significantly affected by treatment (t-test, t_{22} = 0.56, p = 0.577). In Experiment 2, the maximum number of males per colony recorded at any one census date was not significantly affected by treatment (t-test, t_{18} = 1.11, p = 0.28), although there was a trend for this number to be higher in the 25°C treatment (means ± SE: 20°C treatment = 28.5 ± 3.6 males, n = 10; 25°C treatment = 36.0 ± 4.5 males, n = 10; fig. 4.7b). The week of eclosion of the first males by each colony was not significantly affected by treatment (t-test, t_{18} = 0.54, p = 0.595).

Colony thermoregulation

In Experiment 1, the recorded nest temperature of colonies was significantly higher in the 25°C treatment (means ± SE: 20°C treatment = 25.1 ± 0.4°C, n= 12; 25°C treatment = 33.0 ± 04°C; ANCOVA, $F_{1,22}$ = 152.3, p < 0.001) but was not significantly affected by day (ANCOVA, $F_{1,22}$ = 0.724, p = 0.404), or by treatment-day interaction (ANCOVA, $F_{1,20}$ = 1.100, p = 0.173; fig. 4.8a). In Experiment 2, recorded nest temperature was again significantly higher in the 25°C treatment (means ± SE: 20°C treatment = 25.5 ± 0.5°C, n = 19 measurements from 10 colonies; 25°C treatment = 29.3 ± 0.6°C, n = 18 measurements from 10 colonies; LMM, p < 0.001), but not by a day (LMM, p = 0.958), or by treatment-day interaction (LMM, p = 0.496; fig. 4.8b). These findings confirm that our 25°C treatment conditions successfully elevated nest temperature relative to the 20°C treatment conditions.

In Experiment 1, the occurrence of wing fanning behaviour (taken as a binary variable) was highly significantly positively affected by treatment (GLMM, χ^2 = 40.37, p < 0.001), with colonies in the 25°C treatment being much more likely to exhibit wing fanning than colonies in the 20°C treatment (fig. 4.9a and b), but it was not significantly affected by time (GLMM, χ^2 = 0.22, p = 0.643), or treatment-time interaction (GLMM, χ^2 = 0, p = 1). The percentage cover of wax canopy was significantly negatively affected by treatment (Wilcoxon rank sum test, W = 6.5, n = 24, p < 0.001; fig. 4.9c). In Experiment 2, likewise, the percentage cover of wax canopy was significantly negatively affected by treatment (Wilcoxon rank sum tests: day 15, W = 9.5, n = 20, p = 0.001; day 27, W = 12, n = 20, p = 0.002).

Experiment 3 – Oviposition in pre-diapause queens

Based on the data loggers, the mean (± SE) in-box temperature of queens was 14.9 ± 0.1°C for the 15°C treatment and 20.0 ± 0.3°C for the 20°C treatment.

Oviposition

Of the 98 queens in the two treatment groups, only 2 queens exhibited oviposition, both of which were in the 20°C treatment and laid eggs only after the stimulus provided by the addition of a worker. In one of these queens, eggs continued to be laid after worker removal (confirming queen oviposition), but in the other, no further eggs were laid after worker removal. In this latter case, both the worker and queen had active ovaries at

death (confirmed by dissection), so the possibility of worker oviposition could not be excluded. No queens in the 15°C treatment showed signs of oviposition. Over the course of the experiment, more wax nectar cups were produced in the boxes of 20°C queens (9 of 48) than 15°C queens (2 of 48). The construction of these cups is usually a precursor to oviposition (Heinrich 1979); however, all but one of these cups were produced after the introduction of workers, and so may not have been created by the queens.

Diapause

Of the 28 queens in the diapausing treatment, 25 were alive after 65 days (the end of the experiment). As these queens had survived 65 days at approximately 5°C and at least 37 days without food, this is a strong indication that they had entered diapause or a diapause-like state. Although no queens permanently borrowed into the soil, all surviving queens were almost completely inactive at the end of the experiment; this further supports the conclusion that these queens were in diapause and thus that our queens were physiologically capable of diapause.

Discussion

In this study of the effect of temperature on the longevity and productivity of *B. terrestris* colonies, and on the colony foundation behaviour of *B. terrestris* queens, we found no significant difference between 20°C and 25°C on colony longevity in Experiment 1, although we did find the warmer treatment significantly increased colony longevity in Experiment 2 (table 4.1). We found no significant effect of temperature on queen longevity (in Experiments 1 and 2) or on worker longevity (in Experiment 2). In terms of colony productivity, we found that the warmer treatment significantly increased colony size (number of workers) whilst colonies were both growing and declining, with the exception of the growth phase of colonies in Experiment 2, where the effect was not significant but the overall pattern was similar. The timing of the peak colony size did not differ significantly between temperature treatments. In both experiments, the number of new queens produced increased significantly with temperature, but production of males did not change significantly with temperature in either timing or extent. We also found that temperature affected thermoregulatory behaviour, with higher temperature being associated with significantly less wax canopy building in both experiments and a significantly greater probability of wing fanning in Experiment 1.

These findings have several implications. Firstly, they suggest that the effect of higher temperature on colony longevity in *B. terrestris* is either absent or weak. No significant effect of temperature on colony longevity was found in Experiment 1, and the significant positive effect found in Experiment 2 appears largely due to the presence of a few, long-lived workers in the 25^{o}C treatment (fig. 4.4b), since in this experiment there was no significant effect of temperature on the time taken to decline to a colony size of 10 workers. We therefore conclude that, even if higher temperature increases colony longevity, it only does so by a small amount, at least within the temperature range explored by this study. For example, the difference between the mean colony longevities of the two treatments in Experiment 1 was 4 days, with confidence intervals giving a 95% probability that the true increase was between -0.5 and 9.1 days, which is at most equivalent two extra days per degree increase. Thus temperature increase is unlikely to have a marked effect on colony longevity in temperate bumble bees. More specifically, in terms of our first hypothesis, it alone seems insufficient to explain the existence of winter active *Bombus terrestris audax* in southern Britain, which have been observed as much as two months later than usually expected (BWARS, personal communications).

Secondly, our findings show that, surprisingly, temperature did not affect queen or worker longevity, contrary to results of studies of other insects (e.g. Calabi and Porter 1989; Sheehy 2002; Isitan et al. 2010). This suggests that not only colonies, but also individuals within colonies, can be robust with respect to longevity in response to changes in climate. One possible reason for this is that the thermoregulatory ability of colonies is enough to overcome effects of changes in ambient temperature. In support of this, large differences in the thermoregulatory behaviour of workers were found between the treatments, suggesting that colony-level thermoregulation was taking place. Although the different thermoregulatory activities between treatments presumably resulted in different energetic costs, this was not sufficient to produce a discernible difference in longevity. The thermoregulatory behaviours observed did not negate the differences in recorded temperature between the treatments (mean differences between nests in the two treatments were approximately 8^{o}C and 3^{o}C degrees for Experiments 1 and 2, respectively). This is in contrast with the finding from other studies that, within a wide range of ambient temperatures, brood temperature is maintained at approximately 30^{o}C (Heinrich 1979; Weidenmuller et al. 2002). The reason for this discrepancy may be that, in contrast to the other studies mentioned, our data loggers were not positioned directly amongst brood (due to their large size), and

so measured the in-nest, but not brood temperature. In wild colonies where workers forage in the open air and may spend less time in contact with the brood, the effects of temperature on longevity may be more marked. Nonetheless, our results are likely to be applicable to workers spending a large proportion of time in the nest, e.g. nurse workers. Interestingly, although worker longevity was not affected by temperature, we did find a strong effect of worker emergence time on worker longevity, with late-emerging workers having shorter longevities than early-emerging workers. The same pattern has been previously found in other bumble bee species (Goldblatt and Fell 1987; O'Donnell et al. 2000), which has been attributed to increased foraging mortality by late-emerging workers. However, since our colonies were not free-foraging, mortality of foragers by external factors cannot explain this pattern in our study. Other possible reasons for the increased mortality in late-emerging workers could be the increased aggression between workers experienced by colonies late in the colony cycle (the competition phase; Van Der Blom 1986; Bloch and Hefetz 1999; Amsalem et al. 2009), or increased nursing effort by late-emerging workers to increase colony-level reproductive output before colony death at the expense of personal longevity.

Thirdly, given that we found that the timing of the peak of worker production or the timing of first male production did not differ according to temperature in either experiment, our results suggest that colonies do not adjust the timing of their development in response to temperature. This complements the previous finding that timing or duration of field exposure does not alter the timing of colony sexual production in *B. terrestris* (Baer and Schmid-Hempel 2003). If colonies are dependent on an abundance of food at particular stages of development, adhering to a fixed development pattern is likely to be maladaptive in the face of climate change, which may affect the flowering times of plants (Gordo and Sanz 2005; Memmott et al. 2007; Bartomeus et al. 2011), and so may lead to a mismatch in phenology (Miller-Rushing et al. 2010). Although the timing of queen emergence and colony establishment may also vary in response to temperature (Sparks and Collinson 2007; Bartomeus et al. 2011), it is not clear that a fixed shift in the colony cycle will be the most adaptive strategy. As well as phenotypic plasticity, organisms can also react to the environment via natural selection in genetic changes. However, such responses are much slower (since they occur over evolutionary, rather than organismal, time frames), and a low level of phenotypic plasticity may suggest genetic adaptation would be difficult in this case, assuming phenotypic plasticity is a starting point for genetic changes (i.e. genetic accommodation; West-Eberhard 2003; Bell and Robinson 2011).

Fourthly, our findings of a significant positive effect of temperature on the production of queens, and of trends for positive effects on the production of workers and males, suggest that colonies exposed to higher ambient temperatures are able to produce more sexuals, and thus to have higher reproductive success. This is consistent with previous suggestions that the production of new queens in particular is a condition-dependent trait in bumble bees (Owen et al. 1980; Müller et al. 1992; Bourke 1997; Lopez-Vaamonde et al. 2009), since higher temperature colonies had more workers and produced more queens. It is interesting to note that temperature itself was enough to alter productivity, and that changes in food supply were not necessary, since food availability was similar in both of our treatments. The trend for a difference in worker production between treatments, although not significant, may explain the larger colony sizes observed in our high temperature colonies despite no changes in the longevity of workers. Furthermore, as our colonies were only exposed to differences in temperature part-way through colony development, effects on worker and male productivity of treatment may have been dampened because some preliminary investment in these classes of individual had already taken place. Thus colonies reared at high temperatures appear to be more productive than those reared at lower temperatures. This may go some way to compensate any mismatches in phenology generated by a warming climate, although future experiments could help to establish the range of temperatures at which this increase to productivity holds.

Lastly, we were unable to test the effect of temperature on the likelihood of queen oviposition without diapause (second hypothesis) because of the failure of the vast majority of our mated, non-diapaused queens to oviposit. A previous study with *B. t. terrestris* (Beekman et al. 1999) found that 17% of queens began oviposition without diapause, and so the reasons why the queens in our study differed from those in the study of Beekman et al. (1999) are not fully clear. However, Beekman et al. (1999) reared their queens at 29^{o}C rather than 15^{o}C or 20^{o}C and stimulated queen oviposition by adding live honey bee (*Apis mellifera*) workers rather than bumble bee workers, so it is possible that these methodological differences generated the different results, e.g. if a higher temperature is necessary for pre-diapause oviposition. Nevertheless, the fact that queens are capable of ovisposition without diapause (Tasei 1994; Beekman et al. 1999; Goulson 2010; this study) shows that a low-frequency second generation of winter colonies should not be excluded as a possible explanation for winter-active bumble bees. Notably, data on the temporal pattern of relative queen and worker abundance reported during recent winters in Britain are compatible with this putative

phenomenon, because winter peaks of worker sightings have occurred in late December and January, with winter peaks of queen sightings occurring before this time (BWARS, personal communication). This is comparable to the temporal pattern of abundance usually seen in colony foundation in spring and summer (e.g. Prŷs-Jones and Corbet 2011).

In conclusion, the life history responses of eusocial insects such as *B. terrestris* to changing temperature are complex because effects on individuals and the colony may be independent. We here show that individual-level life history may remain stable, perhaps because it is well protected by nest thermoregulation, but that temperature is still greatly important because it can alter reproductive success at the colony level. With regard to the presence of winter-active *B. terrestris audax*, our findings suggest that temperature increases do not increase the longevity, and therefore winter-persistence, of colonies. We also show that increasing temperature alone, within the range of temperatures expected in southern Britain, does not generally render lone mated queens capable of founding colonies without diapause, at least under laboratory conditions. Further studies should focus on investigating the combined effects temperature with other factors, such as resource availability, on colonies in the laboratory. By using carefully controlled studies, in combination with data from the field and models of climate change, it will be possible to construct a fuller understanding of how important pollinators, as well as eusocial insects in general, respond to climate change in nature.

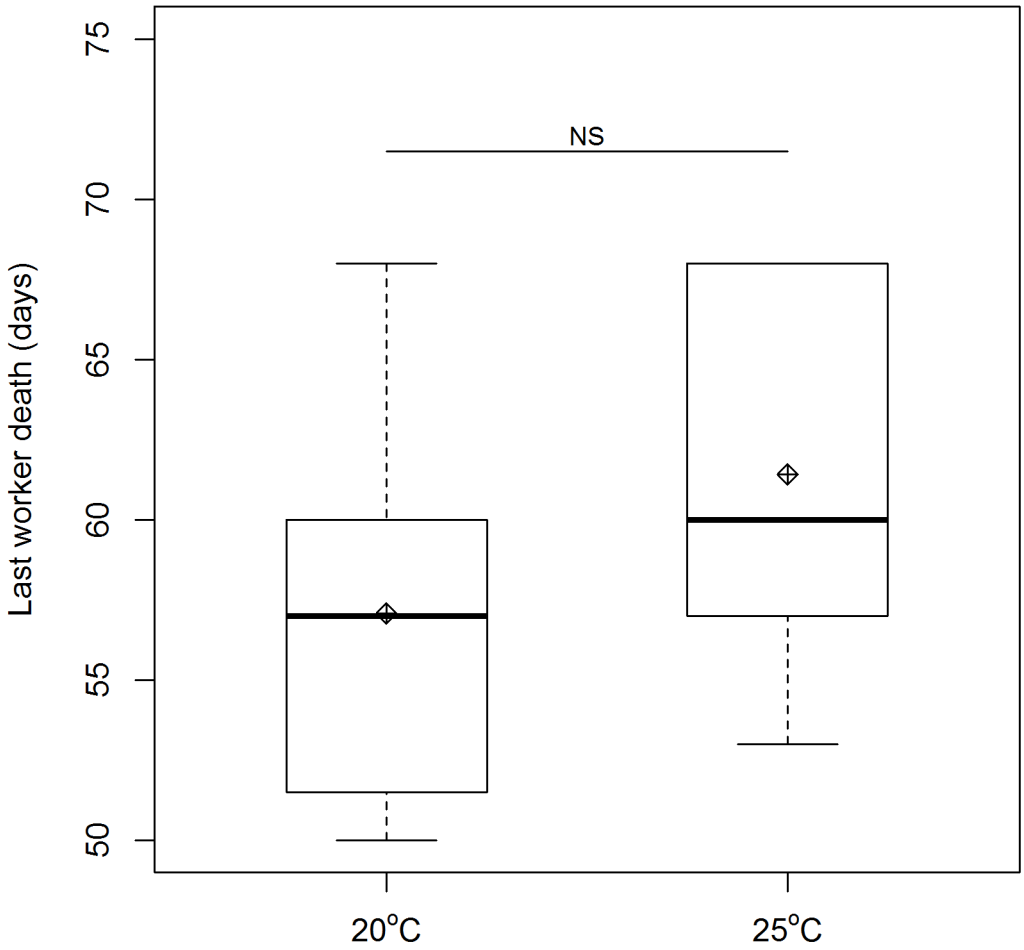

Figure 4.1 Experiment 1: Colony longevity (days between start of experiment and death of last adult worker) of *Bombus terrestris* colonies under 20°C (n = 12 colonies) or 25°C (n = 12 colonies) treatments. Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range, respectively, for each treatment group. NS, not significant (t-test).

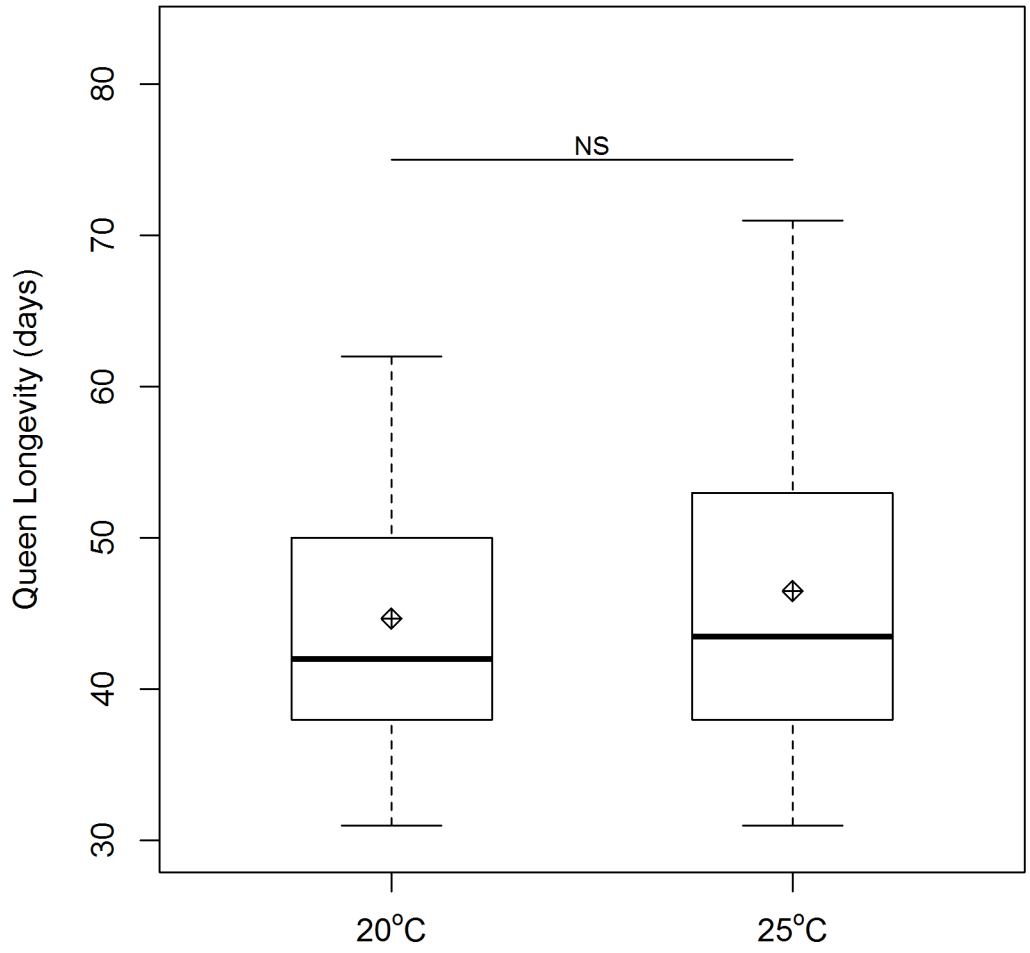

Figure 4.2 Experiment 1: Queen longevity (days between start of experiment and date of queen death) in *Bombus terrestris* colonies under 20°C (n = 12 colonies) or 25°C (n = 12 colonies) treatments. Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range, respectively, for each treatment group. NS, not significant (t-test).

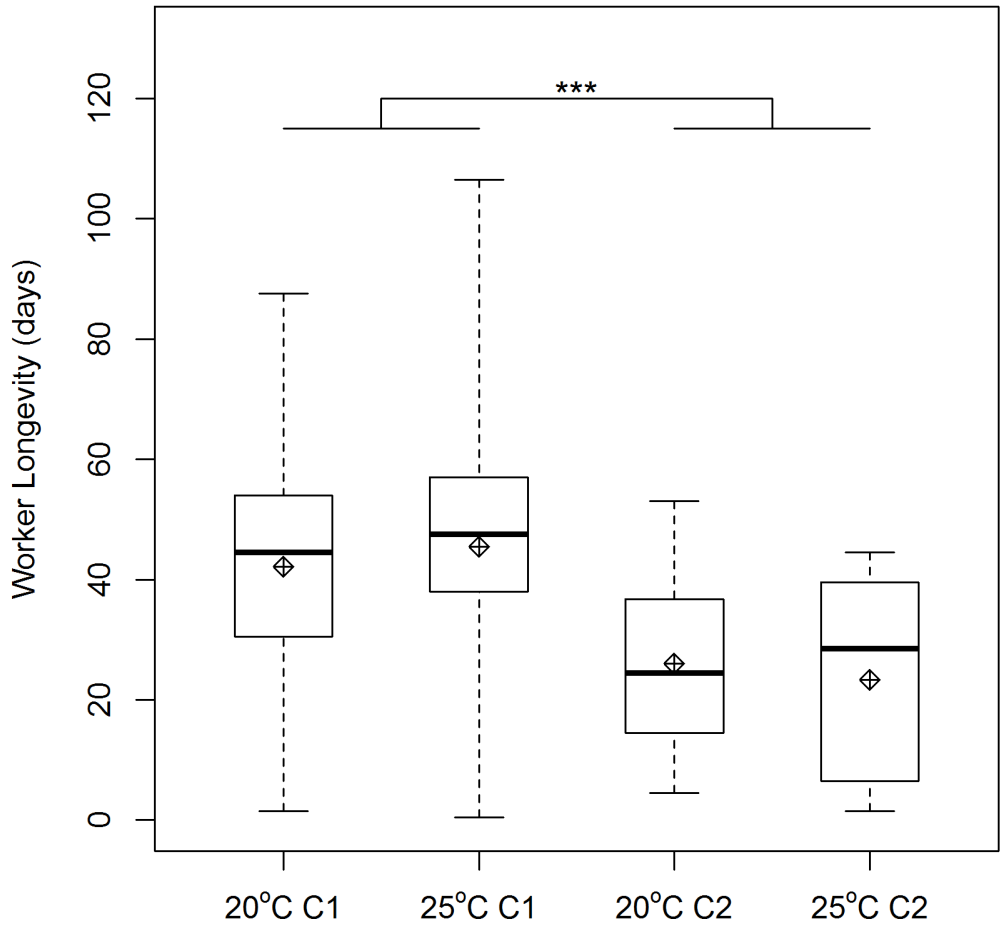

Figure 4.3 Experiment 2: Worker longevity (days between worker eclosion and death) in *Bombus terrestris* colonies under 20°C or 25°C treatments. Boxes: 20°C C1 = contingent 1 workers from 20°C treatment colonies (n = 57 workers from 10 colonies); 25°C C1 = contingent 1 workers from 25°C treatment colonies (n = 66 workers from 10 colonies); 20°C C2 = contingent 2 workers from 20°C treatment colonies (n = 15 from 4 colonies); 25°C C2 = contingent 2 workers from 25°C treatment colonies (n = 22 from 3 colonies). Worker contingent describes the time of eclosion of workers (contingent 1, days 1-15; contingent 2, days 20-30). Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range, respectively, for each treatment group. ***, $p < 0.001$ (linear mixed model).

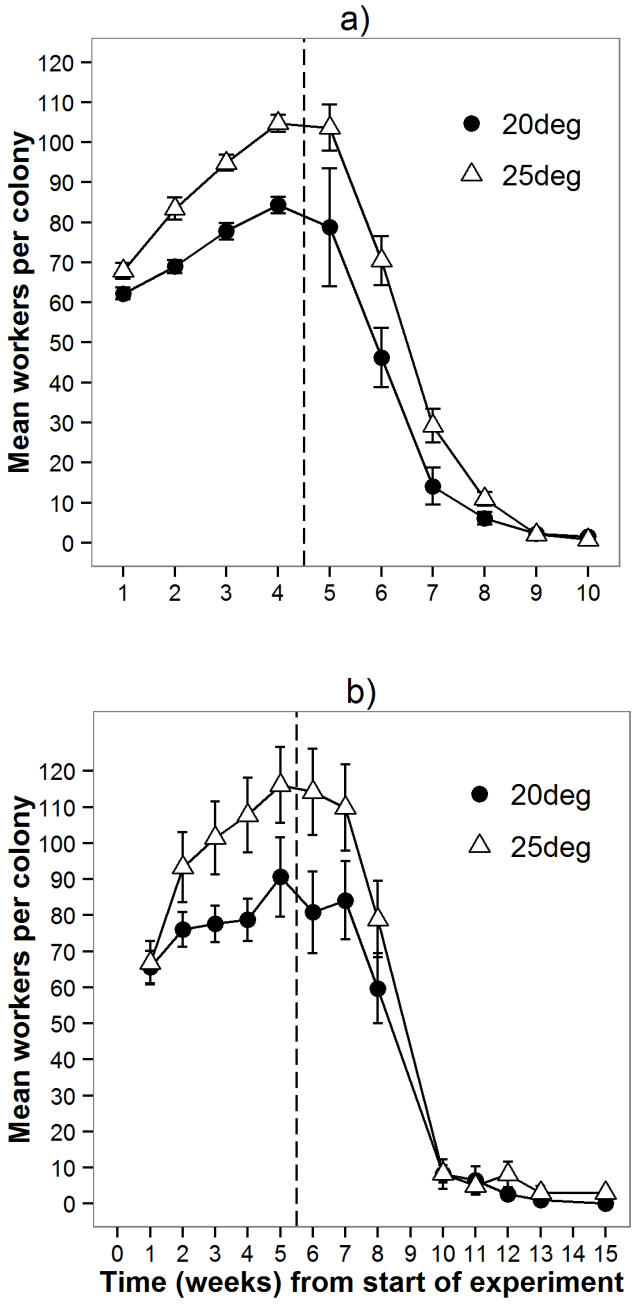

Figure 4.4 Experiment 1 (a) and Experiment 2 (b): Mean weekly number of workers in *Bombus terrestris* colonies under 20°C (n = 12 and 10 colonies for each experiment respectively) or 25°C (n = 12 and 10 colonies for each experiment respectively) ambient temperature treatments. Black circles and white triangles show means for the 20°C and 25°C treatments, respectively, error bars show ± 1 SE. Dotted lines show the division between growth and decline stages for statistical analysis.

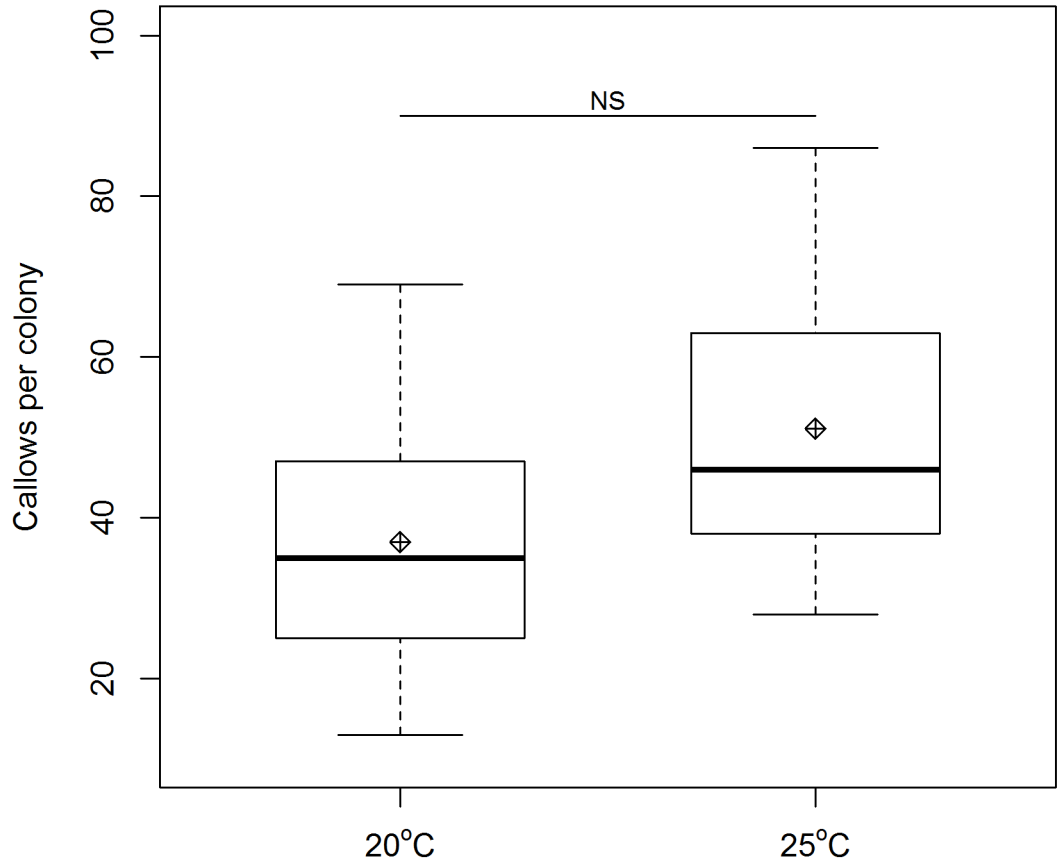

Figure 4.5 Experiment 2: Number of callow (new adult) workers produced in *Bombus terrestris* colonies under 20°C (n = 10 colonies) or 25°C (n = 10 colonies) treatments. Callows were censused daily over days 1-49. Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range, respectively, for each treatment group. NS, not significant (t-test).

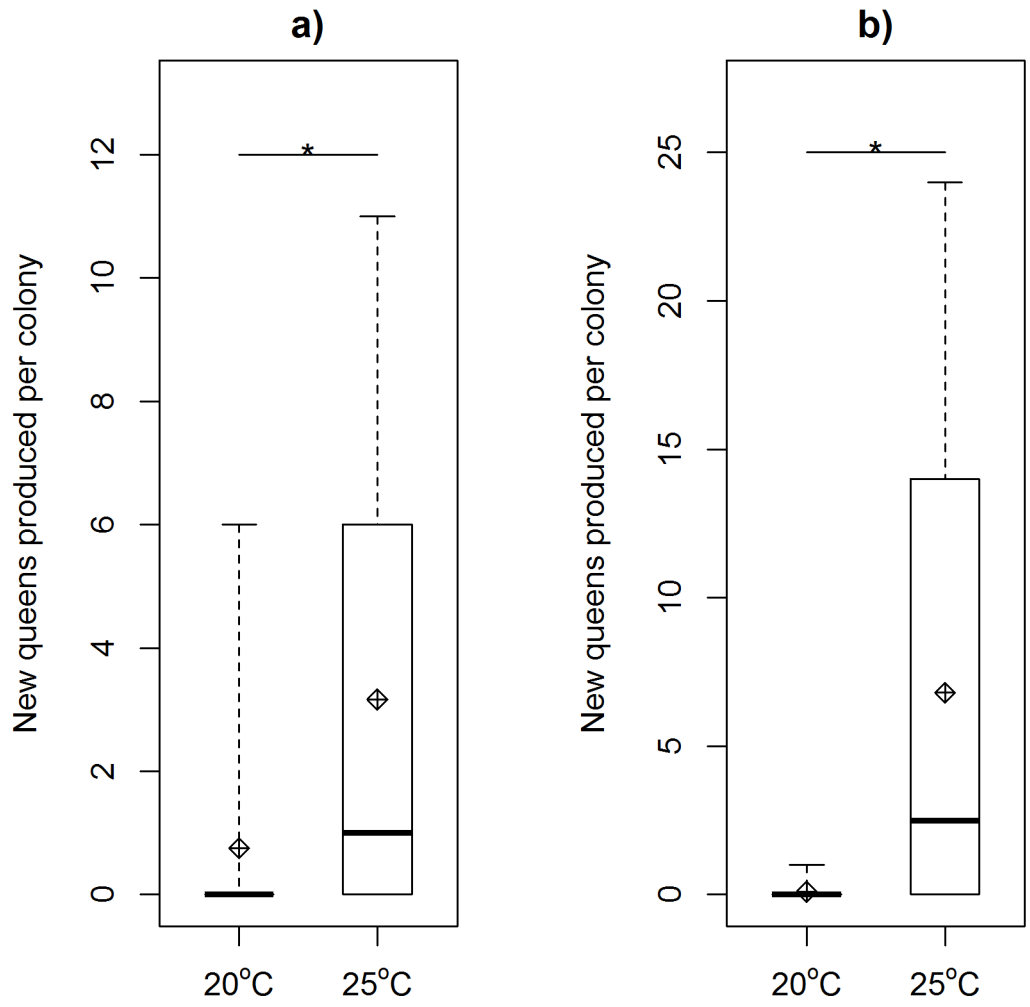

Figure 4.6 Experiment 1 (a) and Experiment 2 (b): Number of new queens produced in *Bombus terrestris* colonies under 20°C (n = 12 and 10 colonies for each experiment, respectively) or 25°C (n = 12 and 10 colonies for each experiment, respectively) treatments. Both figures show the total number of new queens produced by each colony during each experiment. Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range, respectively, for each treatment group. *, $p < 0.05$ (Wilcoxon's signed rank tests).

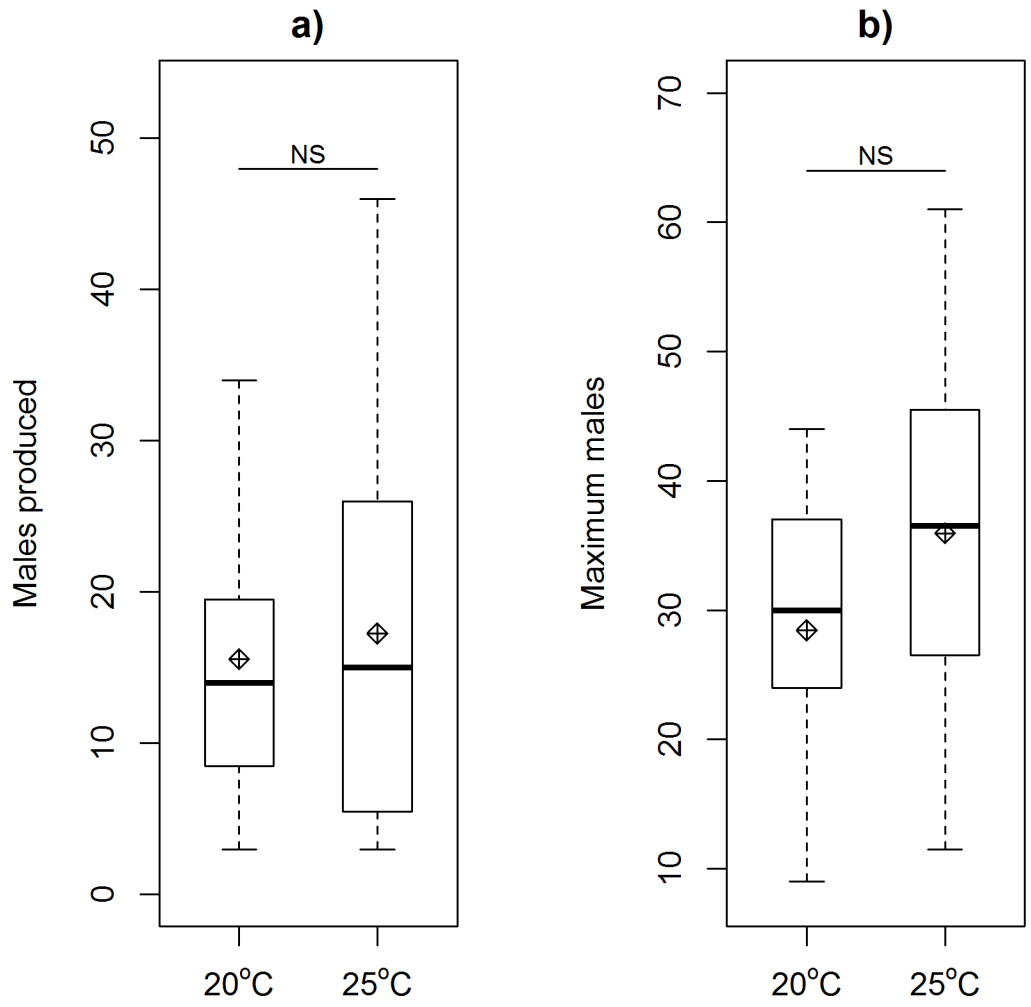

Figure 4.7 Experiment 1 (a) and Experiment 2 (b): Number of males produced in *Bombus terrestris* colonies under 20°C (n = 12 and 10 colonies for each experiment, respectively) or 25°C (n = 12 and 10 colonies for each experiment, respectively) treatments. Figure 4.7a: total number of males censused per colony, and removed, during Experiment 1. Figure 4.7b: highest number of males censused per colony at any one census date (approximately weekly) during Experiment 2. Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range respectively for each treatment group. NS, not significant (t-tests).

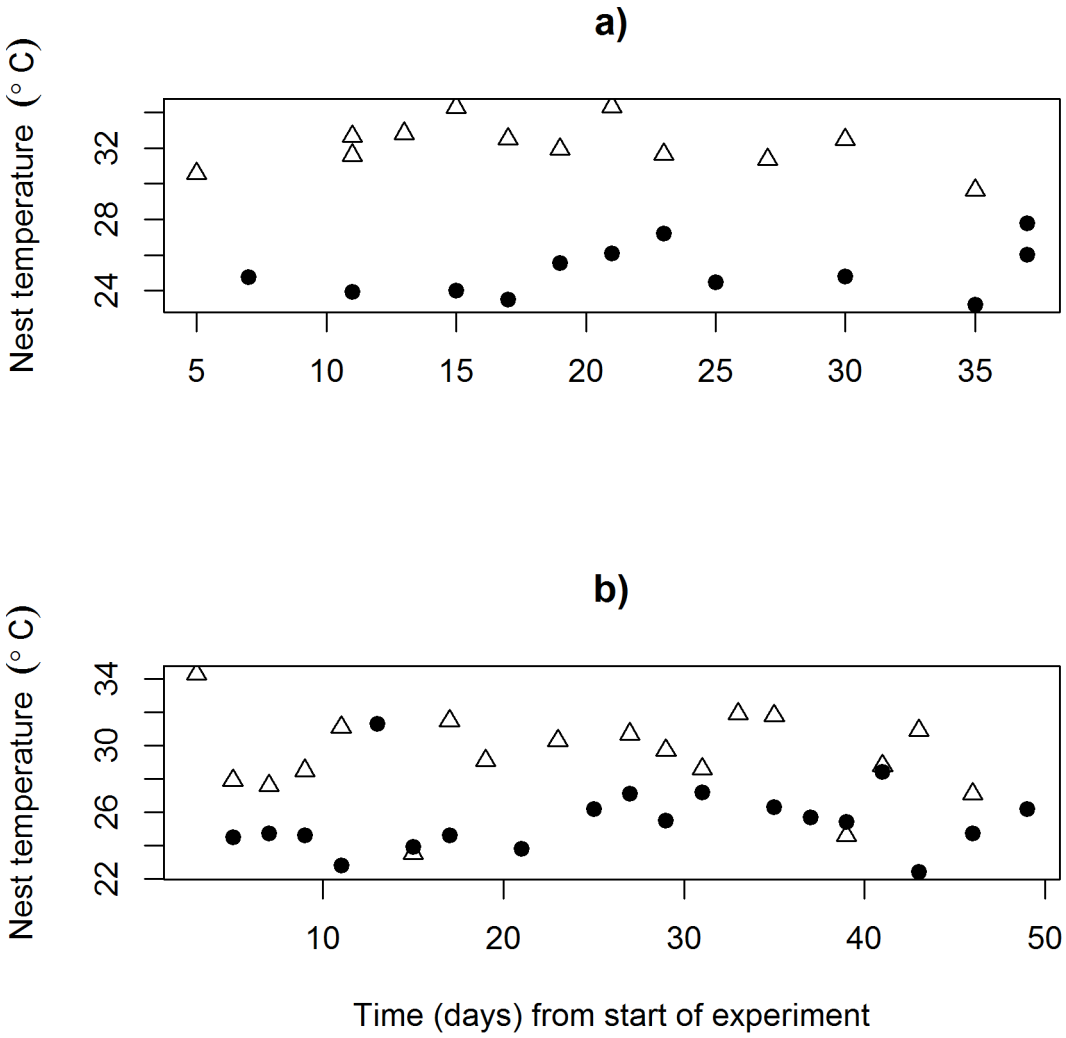

Figure 4.8 Experiment 1 (a), and Experiment 2 (b): Mean nest temperatures recorded during 45 hour data logging periods in *Bombus terrestris* colonies. Black circles show temperatures for colonies exposed to 20°C treatment, white triangles show temperatures for colonies exposed to 25°C treatment. Figure 4.8a: one logging period was conducted in each of 24 colonies (12 periods per treatment) in Experiment 1. Figure 4.8b: one to three logging periods were conducted in each of 20 colonies (19 and 18 periods for the 20°C and 25°C treatments, respectively) in Experiment 2.

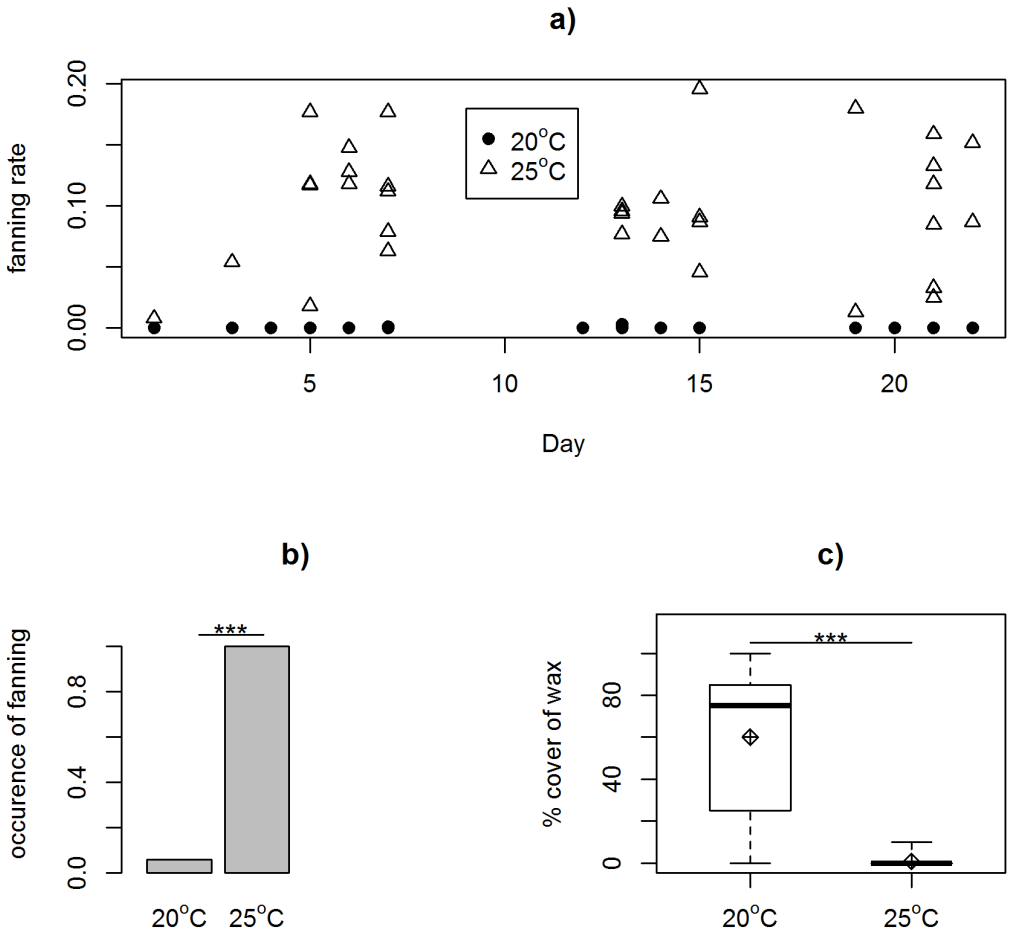

Figure 4.9 Experiment 1: Thermoregulatory behaviour of workers in *Bombus terrestris* colonies under 20°C (n = 12) or 25°C (n = 12) treatments. A 150 x 150 mm section of each colony was filmed for three two hour sessions during the first three weeks of the experiment. Figure 4.9a: mean fanning rate (fanning incidences per worker per minute) for each filming session as a function of time. Black circles and white triangles show means for the 20°C and 25°C treatments, respectively. Figure 4.9b: proportion of filming sessions in which at least one incidence of fanning occurred is shown for each treatment. Figure 4.9c: percentage of each nest covered by a wax canopy, for each treatment on day 29 of the experiment. Diamonds, thick horizontal lines, boxes and whiskers show the mean, median, interquartile range and range respectively for each treatment group. b) ***, $p < 0.001$ (generalised linear mixed model). c) ***, $p < 0.001$ (Wilcoxon rank sum test).

Table 4.1 Comparison of life history traits in *Bombus terrestris* colonies reared at ambient temperature treatments of 15 or 20°C. See *Materials and Methods* for details.

Experiment 1 (n = 12 colonies per treatment)							
Measure	n (20deg, 25deg)	20deg mean	25deg mean	test	Treatment p-value	Time p-value	Interaction p-value
Colony longevity	12, 12	57 ± 6 days	61 ± 6 days	t test	0.673	n/a	n/a
Queen longevity	12, 12	45 ± 9 days	47 ± 12 days	t test	0.624	n/a	n/a
Worker longevity	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Colony growth	12, 12	n/a	n/a	GLMM	*<0.001	*<0.001	0.065
Colony decline	12, 12	n/a	n/a	GLMM	*0.025	*<0.001	*<0.001
Week of peak worker number	12, 12	5 ± 2	6 ± 1	t test	0.267	n/a	n/a
Callow workers produced	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Males produced	12, 12	16 ± 10	17 ± 14	t test	0.899	n/a	n/a
Maximum males at any one census	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Date of first male	12, 12	26 ± 8 days	28 ± 11 days	t test	0.577		
New queens produced	12, 12	1 ± 2	3 ± 4	Wilcoxon	*0.046	n/a	n/a
In-nest temperature	12, 12	25 ± 1 deg	33 ± 1 deg	ANCOVA	*<0.001	0.404	0.173
Rate of wing fanning	12, 12	0.0001	0.0996	GLMM	*<0.001	0.643	1
cover of wax canopy (day 27)	12, 12	60 ± 34 %	1 ± 3 %	Wilcoxon	*<0.001	n/a	n/a

Table 4.1 continued

Experiment 2 (n = 10 colonies per treatment)							
Measure	n (20deg, 25deg)	20deg mean	25deg mean	test	Treatment p-value	Time p-value	Interaction p-value
Colony longevity	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Queen longevity	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Worker longevity	72, 88	39 ± 20 days	40 ± 20 days	LMM	0.624	<0.001	0.618
Colony growth	10, 10	n/a	n/a	GLMM	0.117	<0.001	0.124
Colony decline	10, 10	n/a	n/a	GLMM	*<0.001	<0.001	*<0.001
Week of peak worker number	10, 10	4 ± 2	4 ± 1	t test	0.884	n/a	n/a
Callow workers produced	10, 10	37 ± 17	51 ± 20	t test	0.11	n/a	n/a
Males produced	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Maximum males at any one census	10, 10	28 ± 11	36 ± 14	t test	0.28	n/a	n/a
Date of first male	10, 10	4 weeks	4 weeks	t test	0.595	n/a	n/a
New queens produced	10, 10	0 ± 0.3	7 ± 9	Wilcoxon	*0.015	n/a	n/a
In-nest temperature	10, 10	26 ± 2 deg	29 ± 3 deg	LMM	*<0.001	0.958	0.496
Rate of wing fanning	n/a	n/a	n/a	n/a	n/a	n/a	n/a
cover of wax canopy (day 27)	10, 10	47 ± 33 %	3 ± 6 %	Wilcoxon	* 0.002	n/a	n/a

*statistically significant ($p < 0.05$).

Chapter 5

The role of the foraging gene and queen locomotor behaviour at colony foundation in the bumble bee Bombus terrestris

Abstract

The foraging gene is thought to play an important role in insect foraging behaviour, and may be causally linked to transitions between different behavioural states in eusocial insects. A key, but poorly studied, behavioural transition in many eusocial species is the cessation of queen foraging, which is a component of the shift between the solitary and eusocial stages of the life cycle. A foraging behavioural state is observable under laboratory conditions because it is linked with circadian rhythmicity in locomotor activity and overall locomotor activity. We attempted to induce an early cessation of foraging in pre-laying queens of the bumble bee *Bombus terrestris*, by knocking down the expression of the foraging gene with RNA inactivation (RNAi). Although the knockdown was unsuccessful, we compared foraging gene expression levels in queens varying in age, reproductive status and feeding regime using quantitative PCR. We found that expression was significantly higher in the brains and digestive tracts of laying queens, showing for the first time that the foraging gene is associated with reproductive status in queens. We also found significant effects of feeding regime on expression, but the direction of the effect was different in brains and digestive tracts, suggesting that the foraging gene is involved in multiple responses within the body. Finally, we investigated the behavioural effects of foraging gene expression, age, reproductive status and feeding regime on circadian rhythmicity in locomotory activity and mean locomotory activity, to establish associations with the transition from a foraging state. We confirm previously established behavioural effects according to reproductive status, but found no effects of foraging gene expression, suggesting that this gene does not directly regulate these behaviours in queens. Furthermore, we found behavioural effects of age and feeding regime, suggesting that one, or both, of these factors are involved in the transition from a foraging state in foundress queens.

Introduction

A key challenge in modern biology is linking behaviour with mechanisms of genetic control. One important set of questions concerns how changes in gene expression give rise to behavioural plasticity throughout the lifetime of an individual (Zayed and Robinson 2012). This has begun to be addressed using two key approaches; firstly, the elimination or reduction (knockout or knockdown) of gene expression or translation and the subsequent observation of behavioural effects (e.g. Ben-Shahar et al. 2003; Dawson-Scully et al. 2007; Wang et al. 2010), and secondly, the observation of gene expression differences between organisms exhibiting different behavioural states (e.g. Whitfield et al. 2003; Kent et al. 2009). Although such studies have already yielded large advances in the genetic control of behaviour, particularly in the honey bee *Apis mellifera* (Zayed and Robinson 2012), this area of research is still at an early stage, and a wealth of opportunity exists for exploratory studies to establish which genes influence changes between behavioural states.

One gene with known important associations with behaviour in insects is the foraging gene *for*, which was first characterised as a gene involved in feeding strategies in *Drosophila melanogaster* (Sokolowski 1980; Debelle et al. 1989; Osborne et al. 1997). Various studies have since established a number of associations between *for* and foraging behaviour in other insects, including eusocial insects. In workers of *Apis mellifera*, foraging behaviour is positively associated with *for* expression (Ben-Shahar et al. 2002; Rodriguez-Zas et al. 2012), and increasing activity of the enzyme encoded by *for* (PKG – a cGMP-dependent protein kinase) can stimulate the shift to a foraging phenotype (Ben-Shahar et al. 2002) and induce changes in phototactic activity (Ben-Shahar et al. 2003). This last example suggests a causal link between *for* and inducing a foraging phenotype (Ben-Shahar et al. 2002), although conclusive evidence would require a demonstration that the increased expression of *for* is responsible for an increase in PKG. In the bumble bee *Bombus terrestris*, it has been shown that foraging workers express higher levels of *for* than nurse workers (in-nest workers which do not forage), and that these expression levels decline with age in both of these worker types (Tobback et al. 2011). Although *for* is associated with foraging in eusocial insects other than bees, the directionality of the relationship is reversed in the ants *Pogomomyrmex barbatus* (Ingram et al. 2005) and *Pheidole pallidula* (Lucas and Sokolowski 2009) and in the common wasp *Vespula vulgaris* (Tobback et al. 2008), such that in-nest workers have higher expression of *for* than foraging workers. A similar relationship to these last examples was also found in the workers of a second species of bumble bee, *Bombus*

ignitus (Kodaira et al. 2009), although this may have been confounded by the ages of the workers studied (Tobback et al. 2011).

Despite these associations between *for* and foraging behaviour in eusocial insect workers, almost nothing is known about the genes associated with foraging in colony queens and whether *for* is also implicated. Queen foraging is an essential part of colony foundation in a number of eusocial insect species. This is true of those species where colonies are founded by lone queens (haplometrosis; Cronin et al. 2012), and where nutrition is not provided from queen energy reserves (semi-claustral foundation; Brown and Bonhoeffer 2003; Cronin et al. 2012), requiring queen foraging to feed developing brood. Specifically, this includes many wasps (Ross and Matthews 1991), primitively eusocial bees (Michener 2000) and primitively eusocial ants (Brown and Bonhoeffer 2003). However, when adults start to emerge, foraging for the colony is instead performed by workers. This is presumably adaptive for both the queen and workers for the following reasons: a) if the queen is not foraging, she can devote her time to laying eggs, increasing the worker production of the colony by effective division of labour (Oster and Wilson 1978); and b) foraging carries risks of predation and disorientation, and the queen is a valuable resource to all individuals in the colony, because only she generally has the capability of producing more workers and queens (Brown and Bonhoeffer 2003; Goulson 2010; Cronin et al. 2012). Because this cessation of foraging is a ubiquitous stage in colony foundation among semi-claustral queens and is an important component of the shift from the solitary to eusocial life stage, it represents a key life history event for many species of eusocial insect. Thus, understanding which environmental stimuli and internal mechanisms regulate this transition will yield insights into how eusociality evolved and is maintained in eusocial insects.

Although it is often difficult to measure foraging levels directly in controlled conditions, the measurement of locomotor activity levels in the laboratory is likely to be a reliable proxy for a foraging state, because foraging in eusocial insects has been shown to be associated with circadian rhythmicity in locomotor activity in caged individuals. Such an association is found in workers of honey bees, where foraging workers express circadian rhythmicity, even when kept in constant darkness (Bloch et al. 2001), but nest-bound workers lack circadian locomotor activity even in the presence of light-dark cycles (Bloch et al. 2013). A similar association is also found in workers of harvester ants (Ingram et al. 2009). Furthermore, a non-foraging state is also associated with increased overall activity in honey bee workers (Bloch et al. 2001). In bumble bees,

queens emerging from diapause possess a strong circadian rhythmicity in their locomotor activity, but this rhythmicity is absent in the presence of brood (Eban-Rothschild et al. 2011). Older queens and queens with brood may also be more active overall (Eban-Rothschild et al. 2011). Consistent with the pattern in workers (but reversed in time), these changes are likely to be contemporary with the shift to non-foraging. Thus it is likely that the shift to a non-foraging state by foundress queens (i.e. those founding a colony) is generally accompanied by a reduction or elimination of circadian rhythmicity and increased activity in locomotory behaviour, as in workers.

The proximate stimulus causing the cessation of queen foraging behaviour (including changes in locomotory behaviour), whether or not mediated by changes in *for* expression, is not clear. Brood presence alone may be important, as brood removal or death rescues the rhythmic behaviour of bumble bee queens (Eban-Rothschild et al. 2011), suggesting that brood presence can causally affect circadian rhythmicity in locomotor behaviour. A reversion to foraging behaviour in bumble bee queens is consistent with observations of wild queens foraging if worker numbers become low (Michener 1974, p.323), i.e. after the time at which they should have stopped foraging. However, a lack of rhythmicity can sometimes develop in queens several days before egg laying (Eban-Rothschild et al. 2011), suggesting that there may be a more complex relationship between foraging and reproductive state (Eban-Rothschild et al. 2011; Bloch et al. 2013). Furthermore, reproductive behaviour in bumble bee queens is negatively associated with brood feeding behaviour, which is a further change in queen behaviour associated with the shift to the eusocial life stage, but which does not occur until after the emergence of adult workers (Woodard et al. 2013). Queen reproductive state has also been shown to be associated with foraging in the harvester ant *Pogonomyrmex californicus*, as foraging queens tend to have lower ovary activation (Dolezal et al. 2013). Other conceivable factors influencing the cessation of foraging could be age, or the available provision of food. Foraging behaviour is affected by age in a number of eusocial hymenoptera, such honey bees (Robinson et al. 1994) and various ant species (Bourke and Franks 1995). In the case of queen foraging cessation, age (or time) could be a proxy for the optimal stage to cease foraging; such a response of bumble bee queens to time, rather than social environment, has already been implicated in the colony-level onset of male production (Holland et al. 2013). The available provision of food in the nest could also be important because low nest food levels have been shown to increase the propensity to forage in workers of honey bees

(Camazine 1993; Ament et al. 2010) and the ant *Lasius niger* (Mailleux et al. 2010) and ample resources may signal an appropriate time to cease foraging.

Due to the association of foraging behaviour with circadian rhythmicity of locomotor activity and overall locomotor activity levels in queens, it is possible that expression levels of *for* will be related to these behaviours. This is plausible mechanistically, because *for* is thought to mediate foraging in honey bees by modulating phototaxis (Ben-Shahar et al. 2003), which may be linked to circadian rhythmicity. Furthermore, unpublished data from *B. terrestris* have also shown that *for* is expressed at higher levels in confined, asocial queens (with brood removed) than in social queens (Almond et al, unpublished data), which is consistent with a positive association between foraging behaviour and *for* expression.

One way to test for the function of a specific candidate gene is to use RNAi (RNA interference) (Taylor and Francis 2010), which is a technique allowing the knockdown (reduction in translation) of a specific gene using double-stranded RNA strands (dsRNA) (Fire et al. 1998). Past studies have revealed that it is possible to induce RNAi through the oral ingestion of double stranded RNA in arthropods (Timmons and Fire 1998; Araujo et al. 2006; Patel et al. 2007; Garbian et al. 2012). Here, we used this technique in non-laying queens of *Bombus terrestris* in an attempt to determine whether a reduction in *for* translation induces a premature shift to the arrhythmic, more active, state thought to be associated with non-foraging queens that successfully produce adult workers. Although the attempted RNAi failed to produce any changes in *for* expression as hoped, we were able to test whether reproductive status, age or feeding regime, all of which may be associated with the shift to foraging behaviour, affected *for* expression. In addition, we were also able to use the *for* expression data to establish whether *for* expression, reproductive status, age or feeding regime affected locomotor activity.

Materials & Methods

Queen rearing

Mated and post-diapause *Bombus terrestris audax* queens (n = 199) were obtained from a commercial supplier (Biobest, Westerlo, Belgium) on 24 January 2013. Queens were kept in individual plastic boxes (140 x 79 x 60 mm) and supplied with ad libitum sugar solution (50% water/50% syrup; Koppert, Berkel en Rodenrijs, Netherlands) and

pollen. All queens were housed in a 28^{o}C and 60% RH controlled environment room under constant red lighting conditions.

Effect of RNAi on locomotor activity

Tracking of locomotor activity

Beginning on 13 March 2013, the effect of RNAi treatments on locomotor activity levels was tested using a random sample of non-laying queens (n = 46; table 5.1 'treated queens'). Each day, 8 random non-laying queens were selected to begin treatment (3 with *for* dsRNA treatment, 2 with *gfp* dsRNA treatment, 3 with water treatment; see 'RNAi treatment'), except when this would mean > 30 queens being monitored simultaneously (due to limitations with activity tracking). The locomotor activity of all treated queens was measured using the Object Tracker freeware (available on the Internet at http://iEthology.com/), which logs the positions of individuals each second in real-time, allowing the calculation of locomotor activity levels of each individual. Preliminary tests revealed that this method accurately captured queen activity on foot (queens in boxes did not fly under red light conditions), and Object Tracker has been previously shown to capture movement at smaller spatial scales than more traditional activity tracking (Donelson et al. 2012). Multiple queens (up to 30) were tracked simultaneously using a webcam whose field of view incorporated all focal individuals. Opaque cardboard dividers were placed between individual boxes during tracking in order to prevent locomotor activity being affected by visual stimuli of other bees.

Each day, individuals were tracked for 20 hours, beginning at 1700-1745 and ending at 1300-1345 on the following day. In order to minimise disturbance during tracking, the controlled environment room was only entered during the tracking downtime (i.e. when tracking was not being carried out), between 1300-1345 and 1700-1745 each day (depending on when tracking occurred). This downtime was used for treatment (see 'RNAi treatment') and for daily randomisation of tracking positions of boxes. Tracking of each treated queen was performed over a 10-day period, i.e. 10 tracking sessions, except where queens died before this time (n = 6, not included in sample size). The positional data gained from tracking were used to estimate the rate of locomotor activity by finding the euclidean distance between the position of each individual at each time point and at the following time point. These values were then used to find the total estimated distance travelled per minute. Due to the sensitivity of the software, there was an inherent error associated with detecting movement created by constant tiny fluctuations in light and shadow. In order to correct for this error, 2-4 dead (and

therefore static) queens were tracked on each day tracking was performed. These queens were boxed in the same way as the living tracked queens, as described above. The mean movement rate recorded for these queens was considered to represent the minimum threshold for detecting movement, and therefore this value was subtracted from all movement rates in tracked queens, as in other studies (e.g. Eban-Rothschild et al. 2011).

Table 5.1 Sample sizes and treatments of *Bombus terrestris* queens in the experiment (for details, see *Materials & Methods*).

	Early queens	Trial queens	Laying queens	Treated queens	Late queens
Sample size	10	17	7	46	10
Feeding regime	ad libitum	ad libitum	restricted (6) or ad libitum (1)	restricted	ad libitum
Age range (day of dissection/end of observation)	8	15-43	22-62	59-72	75
Data collected	expression only	behaviour only	expression and behaviour	expression and behaviour	expression only

Production of dsRNA

The use of RNAi requires the production of double stranded RNA (dsRNA) complementary to a region of the transcript (mRNA) of the focal gene. When present in cell cytoplasm, this dsRNA is cleaved by the RNAse III-type enzyme, 'Dicer', into small interfering RNA (siRNA) duplexes (roughly 21 nucleotides in length). The individual strands of the duplex separate and the antiscence strand (targeting the mRNA) is incorporated into a multi-protein complex known as the RNA induced silencing complex

(RISC). From there, the ssRNA guides RISC to the target mRNA which normally results in its degradation or inactivation. As well as producing dsRNA for the *for* gene (the gene to be knocked down), dsRNA for another gene, *gfp* (green fluorescent protein), was also produced. Since bumble bees do not possess the *gfp* gene, the *gfp* dsRNA acted as a nonsense sequence treatment, which we used to control for any additional effects of dsRNA presence (see 'RNAi treatment'). For each gene, dsRNA was produced using (in brief) the following steps: 1) identification and amplification of the target sequence; 2) molecular cloning of the target DNA sequence to produce a stock of DNA with the target sequence; 3) transcription of the DNA to produce target RNA strands; and 4) binding of the ssRNA strands to create dsRNA. This methodology is similar to that described in other sources (e.g. Maori et al. 2009), but, for clarity, each step is explained in turn here: 1) the target transcribed region of the gene was identified using the genome browser function on the National Centre for Biotechnology Information (NCBI) website. We extracted DNA from *Bombus terrestris* larvae using a DNeasy kit (Qiagen, Hilden, Germany) and PCR amplified the target sequence. Four primers were used: a pair of primers (forward and reverse) with attached T7 promotor sequence, and a pair of primers without this sequence. For the foraging gene, the primers were designed for a 400bp region of the largest exon of the foraging gene (exon7). For the *gfp* gene, pre-existing primers were utilised (for primer sequences for both genes, see table 2). In each reaction, a T7-containing primer was paired with a non-T7 primer. Thus, (for each gene separately) we amplified two sequences, one with a T7 promoter on the 3' end and the other with the promoter on the 5' end. The products of these reactions were cut from a 1.2% agarose gel using a Zymoclean Gel DNA recovery kit (Zymo Research, Irvine, USA). This step was repeated with T7 containing primers targeting GFP from the plasmid pEGFP-N1(Clontech) 2) The extracted strands were replicated using molecular cloning with a pGEM-t vector in DH5-alpha transformation competent *E. coli* host cells and extracted from the host using a miniprep kit (Qiagen). The correct sequence of the resultant plasmids was confirmed by Sanger sequencing. 3) The resultant DNA strands (both complementary strands separately for each gene) were transcribed in vitro using T7-dependent RNA polymerase (MEGAscript RNAi kit, Invitrogen, Carlsbad, USA). 4) The complementary RNA strands were annealed by mixing the two products, heating them at 75 degrees and cooling them to room temperature. The concentration of the dsRNA was calculated on a nanodrop and the band integrity of the RNA was checked on a 1.2% agarose gel to ensure the products were not degraded. In order to introduce dsRNA into the queens, we supplied the dsRNA in liquid food (see Treatment and activity tracking),

because previous studies with various arthropods, including honey bees, have shown that the oral ingestion of dsRNA can be sufficient to induce RNAi (Timmons and Fire 1998; Araujo et al. 2006; Patel et al. 2007; Garbian et al. 2012). To prevent premature degradation, each dsRNA preparation was kept at -20^{o}C until the day of use.

RNAi treatment

As described above (see *Tracking of locomotor activity*), treatments were applied to queens in the downtime before each period of tracking. Treatments used were one of three types: i) syrup solution containing *for* dsRNA; ii) syrup solution containing *gfp* dsRNA to control for the effect of dsRNA; and iii) syrup solution only. Treatment solutions were provided in small (250µl) eppendorf tubes which were cut down to size, allowing access by the bees' tongues, and then fixed to the pollen food mixture to prevent movement of the tubes in the box. The treatment solutions consisted of 15 or 21µl of pure sugar syrup and an equal amount of one of the following, depending on treatment group: i) water containing 5µg of *for* dsRNA; ii) water containing 5µg of *gfp* dsRNA; or iii) water only. In this way, the sugar concentration of the treatment solutions was kept equal to that in the bee's normal diet (i.e. 50% water/50% syrup). In order to increase the likelihood that the treatment solution was ingested, the queens were given no other syrup at the same time, restricting their food intake. Before tracking began each day, we recorded whether the treatment solution had been consumed, and all treatment queens were then given ca 500µl of sugar solution for the tracking period. This quantity was found to be sufficient to ensure that queens did not die due to starvation, but still generally consumed all of the treatment solution during the tracking downtime (approx. 90% consumption rate).

Dissection and qRT-PCR

All treated queens were dissected after 10 complete days of tracking and treatment in order to remove brains and digestive tracts (from honey stomach to rectum). Queens were chilled on ice prior to dissection (to facilitate live dissection), which occurred ≤ 7 hours after the end of their final tracking period. The removed organs were preserved in RNA later and stored at -20^{o}C until qRT-PCR to prevent RNA degradation. qRT-PCR of treated queens allowed for testing of whether the knockdown of *for* was successful. Brains were selected because we assumed any behavioural effects of activity resulting from *for* knockdown would result from its occurrence in the brain. We also selected the

digestive tract because we assumed that successful knockdown would be most likely in the digestive tract (given that the dsRNA was ingested). We extracted totalRNA from the stored tissue samples by homogenising them with Trizol reagent (Invitrogen). We then separated the organic phases with chloroform, precipitated the RNA in isopropanol, and resuspended the RNA in RNase-free water. The samples were treated using the Invitrogen TURBO DNAfree kit to remove any residual DNA that had carried over from the RNA extractions. The totalRNA was then reverse transcribed into cDNA using the Omega GoScript reverse transcription system with a universal primer (T(20)VN, table 2). The cDNA was used to perform qPCR, which allows the quantification of the copy number of specific mRNA strands. From each sample, qPCR was performed for three genes: *for*, *ArgK* and *PLA2*, with the latter two used as reference genes, which have been shown to be relatively stable in bumble bees (Hornakova et al. 2010). The qPCR was performed by an external company (qStandard, Division of Surgery & Interventional Science, University College London, UK), using pre-designed (*PLA2*) or specially designed probe sequences (*for* and *ArgK*; table 2). Copy numbers of *for* were obtained from 2µl of cDNA, derived using standard curves (Rotor Gene software, Qiagen) and then divided by the geometric mean of the two reference genes as a normalisation factor for each sample. This provided an adjusted copy number used to represent expression.

Effects of age, reproductive status and feeding regime

Prior to measuring the activity of treated non-laying queens, another group of non-laying queens were randomly selected for activity tracking as a preliminary trial (n = 17; table 1 'trial queens'). Beginning on 7 February 2013, these queens underwent tracking using the same procedure as for treated queens, except that they did not receive treatment (they were fed ad libitum with sugar solution) and they were tracked for 7-10 days each (excluding queens dying before this time, n = 3, not included in sample size). These queens were not dissected and so no expression data were gained from these queens. In addition, between 6 February 2013 and 6 March 2013 we selected laying queens for treatment and tracking (n = 6; table 1 'laying queens'). All queens detected laying eggs during this period were randomly assigned to one of the above three treatments (i.e. fed with for dsRNA, gfp dsRNA or water plus syrup) and were also randomly selected to either receive living pupae (supplied from mature conspecific colonies) or not to receive pupae. Such queens were treated and tracked for seven days (immediately following the day on which the first egg was detected) using the same method as described for treated queens. The aim of this procedure was to

determine whether *for* knockdown and/or addition of pupae affected the locomotor behaviour of egg-laying queens. However, a low frequency of laying queens within the experimental time period resulted in an initial sample size of 10 laying queens, with only six surviving to the end of the tracking period. A further single egg-laying queen was detected on 15 March, and this queen was fed ad libitum and tracked for 10 days. All seven surviving tracked laying queens were dissected for qRT-PCR in the same way as treated non-laying queens. Finally, in order to gain more *for* expression data from queens at different ages, a further 10 at the start of the experiment and 10 at the end (table 1 'Early queens' and 'Late queens' respectively) were also randomly selected for and dissected for qRT-PCR in the same way as treated non-laying queens. Because our queens therefore varied in age, reproductive status and feeding regime (i.e. ad libitum for non-treated queens and restricted for treated queens), this allowed us to compare expression amongst these different groups of queens and also measure how they differ in locomotor activity. Whether or not queens were given treatment should not itself have had an effect on expression or locomotor activity, beyond the effect of differences in age, laying status and feeding regime, since dsRNA was shown to have no effect (see 'Results').

Statistical analysis

To analyse queen activity rates, the Euclidean distance between the positions of each queen at each time point and the subsequent time point was calculated. This allowed the rate of movement for each minute, during which a queen was being tracked, to be estimated. The mean rates of movement per minute for each queen were used as a measure of activity. In addition to using the overall activity of queens, we also used Lomb-Scargle periodograms in order to calculate whether each queen had significant circadian rhythmicity and the length of the period of any such rhythmicity. Lomb-Scargle periodograms were chosen because they do not require time series data to be evenly spaced (Ruf 1999), and therefore allowed for the missing data from the tracking down-periods. Since periodograms using the mean activity rate for each minute were oversaturated, i.e. showed significant periodicity at almost every time interval, models were created using data from approximately one minute per tracked hour from each queen (200 random minutes per queen), which yielded a single clear peak of periodicity, or otherwise no peak, for each individual (Bloch et al. 2001). Queens showing a peak of periodicity between 20 and 28 hours, significant at $p<0.01$ (Ruf 1999), were classed as having circadian rhythmicity (hereafter, rhythmicity).

Effect of RNAi on locomotor activity

In order to evaluate the effects of treatment on locomotor activity and *for* expression in treated queens (n = 46), several ANOVA linear models were used. Specifically, treatment was used as a predictor variable, with mean locomotor activity, *for* expression in brains or for expression in digestive tracts,used as the response variable in each model respectively. Separate models were instead of a MANOVA because all three variables were found not to be correlated (separate Spearman rank or Pearson's product moment correlations). Two outlier queens (both water-treated) with particularly high brain *for* expression (greater than 3 SDs from the mean) were excluded from the model with brain *for* expression as a response variable. The effect of circadian rhythmicity in locomotor activity was not analysed statistically as no variation was present (see *Results*).

Effects of age, reproductive status and feeding regime on for expression

A second set of analyses was used to determine the effect of age, laying-status and feeding regime on *for* expression in both brains and digestive tracts in all queens with expression data (n = 72). Two linear models were produced with age (time of dissection in days after receipt), laying status and feeding regime as predictor variables, and with brain *for* expression and digestive tract *for* expression as the response variable for each model, respectively. To check for system-wide correlations in *for* expression, the relationship between brain and digestive tract expression was tested using Pearson's product moment correlation coefficent.

Effects of for expression and other factors on locomotor activity

A third set of analyses was used to determine the effects of *for* expression (irrespective of treatment), age, reproductive status and feeding regime on locomotor activity. A generalised linear model with a binomial error distribution and logit link function was produced with brain and digestive tract *for* expression levels as predictor variables and the probability of circadian rhythmicity (*p*-value from Lomb-Scargle periodogram) as the response variable. A chi-squared test was used to compare the number of rhythmic queens among ad libitum fed, non-laying queens; food-restricted, non-laying (treated) queens; and food-restricted laying queens. Post hoc chi-squared tests were then used to compare each group independently. In order to compare rhythmicity across all queens, some of which were only tracked for 7 days, a second set of rhythmicity p-values were calculated based only on the final 7 days of tracking for each queen and

used for these comparisons. A linear model was produced with brain *for* expression, digestive tract *for* expression, age, and laying status as predictor variables, and with activity as the response variable. Because of a limited sample size, only main effects and two way interactions were included as terms in the model. Since *for* expression for either tissue was not a significant predictor of activity (see *Results*), allowing the inclusion of non-dissected queens, a linear mixed model was produced to test the fixed effects of age and laying status on activity, with queen as a random effect (to account for 6 queens which were tracked once as non-treated queens and then again as treated queens). This analysis excluded feeding regime, because it had a high correlation with age (Pearson's r = 0.82), since all older queens with behavioural data had a restricted feeding regime (table 1, treated queens). Finally, in order to test the relationship between mean locomotor activity and rhythmicity, a Spearman rank correlation was used between mean locomotor activity and the probability of having arrhythmicity in the final 7 days of tracking (p-value from Lomb-Scargle periodogram).

Results

Effect of RNAi on locomotor activity

Amongst treated nonlaying queens, the mean complete consumption of treatment solution per day was 88%. Queens consuming less than half their total dosage of treatment across days (i.e. consuming <5 doses; n = 4) were excluded from all analyses comparing between treatments. Treatment had no significant effect on the proportion of rhythmic queens, since all treated queens had significant circadian rhythmicity (n = 42). Similarly, treatment had no significant effect on overall activity (means ± SE: *for* queens = 2.7 ± 0.4; *gfp* queens = 3.0 ± 0.2; water queens = 2.7 ± 0.1; ANOVA, $F_{2,39}$ = 0.59, n = 42, p = 0.558). Furthermore, treatment had no effect on *for* expression in either tissue studied (Brains, means ± SE: *for* queens = 2244 ± 56 copies; *gfp* queens = 2054 ± 46 copies; water queens = 2114 ± 72 copies; ANOVA, $F_{2,36}$ = 1.80, n = 39, p = 0.180. Digestive tracts, means ± SE: *for* queens = 12933 ± 766 copies; *gfp* queens = 10133 ± 874 copies; water queens = 11219 ± 914 copies; ANOVA, $F_{2,39}$ = 2.34, n = 42, p = 0.110; fig. 5.1).

Effects of age, reproductive status and feeding regime on for expression

There was no significant effect of age on brain *for* expression (ANOVA, $F_{1,63}$ = 2.39, n = 68, p = 0.123) or digestive tract *for* expression (ANOVA, $F_{1,66}$ = 0.02, n = 72, p =

0.878). Laying queens had significantly higher expression than non-laying queens both in brains (means ± SE: laying queens = 2453 ± 113, n = 6; non-laying queens = 2240 ± 36, n = 62; $F_{1,63}$ = 6.44, p = 0.014; fig. 5.2a) and digestive tracts (means ± SE: laying queens = 15492 ± 1189, n = 6; non-laying queens = 10816 ± 401, n = 66; $F_{1,69}$ = 13.03, p < 0.001; fig. 5.2b). Queens fed ad libitum had significantly higher expression than queens with a limited food supply in brains (means ± SE: ad libitum queens = 2435 ± 69 copies, n = 20; limited queens = 2180 ± 37 copies, n = 50; $F_{1,63}$ = 14.21, p < 0.001; fig. 5.3a), but there was a significant opposite effect in digestive tracts, in which queens fed ad libitum had significantly lower expression than queens with a limited food supply (means ± SE: ad libitum queens = 9000 ± 376 copies, n = 20; limited queens = 12055 ± 500 copies, n = 52; $F_{1,69}$ = 10.26, p = 0.002; fig. 5.3b). In brains, there was a significant interaction between age and feeding regime, with expression increasing with age in queens fed ad libitum, but decreasing with age in queens with a limited food supply ($F_{2,63}$ = 3.25, p = 0.045; fig. 5.4a). No such interaction was found in digestive tracts ($F_{2,67}$ = 0.04, p = 0.958; fig. 5.4b). There was no significant interaction between age and laying status on expression in either brains ($F_{1,62}$ = 0.15, p = 0.703) or digestive tracts ($F_{2,66}$ = 0.72, p = 0.490). Furthermore, no relationship was found between *for* expression levels in brains and digestive tracts across all queens (Pearson's product moment correlation, r = -0.03, n = 70, p = 0.814).

Effects of for expression and other factors on locomotor activity

In testing the effects on circadian rhythmicity, no significant effect on the probability of rhythmicity was found for brain *for* expression (generalised linear model; χ^2 = 0.35, n = 70, p = 0.551), gut *for* expression (χ^2 = 0.65, p = 0.422) or an interaction (χ^2 = 0.39, p = 0.528). The proportion of rhythmic queens differed significantly amongst ad libitum fed, non-laying queens; food-restricted, non-laying (treated) queens; and food-restricted laying queens (chi-squared test, χ^2 = 40.3, df = 2, p < 0.001; fig.5.5). All food-restricted non-laying queens (n = 46) were rhythmic, but only 43% of food-restricted laying queens (n = 7) and 29% of ad libitum fed, non-laying queens (n = 17) were also rhythmic. Pairwise comparisons revealed that the proportion of rhythmic queens in food-restricted, non-laying (treated) queens was significantly higher than both food-restricted laying queens (chi-squared test, χ^2 = 20.8, df = 1, p < 0.001) and ad libitum fed, non-laying queens (chi-squared test, χ^2 = 35.7, df = 1, p < 0.001), but that there was no significant difference between the latter two groups of queens (chi-squared test, χ^2 = 0.03, df = 1, p = 0.874). There was no significant effect on overall activity of *for* expression in either brains (ANOVA, $F_{1,40}$ = 0.12, n = 50, p = 0.731; fig.5.6a) or

digestive tracts ($F_{1,45}$ = 0.76, n = 52 p = 0.388; fig.5.6b). However, a significant negative effect was found of age (linear mixed model, χ^2 = 101.5, n = 70, p <0.001; fig.5.6c). A trend of greater activity in laying queens was seen, but this was not significant (χ^2 = 2.18, p = 0.140; fig.5.6d). Although feeding regime was highly correlated with age (see *Materials & Methods*), the pattern of decline in activity with age was apparent within either feeding regime (fig. 5.6c). Mean activity rate and the probability of being arrhythmic were highly significantly positively correlated across all queens (Spearman rank correlation, ρ = 0.70, n = 70, S = 17058, p < 0.001). No significant interaction effects were found. Actograms depicting examples of the activity levels of rhythmic and arrhythmic queens are shown in fig. 5.7. The activity peaks in rhythmic queens always shortly followed the tracking downtimes (e.g. fig. 5.7a), suggesting that some factor (e.g. the burst of white light upon entering the room, or a small change in temperature) at these times was sufficient to entrain rhythmicity in these queens. Nonetheless, the rhythmic activity patterns are not likely to be a simple response to disturbance because: a) the peak of activity usually followed the disturbance by several hours, and declined very gradually over time; and b) some (arrhythmic) queens showed no marked response to disturbance (e.g. fig. 5.7b).

Discussion

Comparison of *for* expression levels amongst treated queens showed that our dsRNA treatment did not knockdown the gene (i.e. reduce mRNA) in either digestive tracts or brains and so, unsurprisingly, no effects of the RNAi treatment on behaviour were seen. Although ingested dsRNA may not have reached the brain, it would seem surprising if the dsRNA was not at least taken into the digestive tract, since RNAi by dsRNA ingestion has been demonstrated in other insects. Therefore, it is not clear why *for* expression was not knocked down in either tissue, although it may have been because the amount of dsRNA used was insufficient and/or RNAi by oral dsRNA ingestion is, for other reasons, not possible in adult bumble bees (since this was, to our knowledge, the first study attempting to utilise the technique in bumble bees). Otherwise, it may have been that *for* expression was knocked down, but then increased again in the time between the final dsRNA treatment and dissection (approximately 25 hours). This last explanation is not likely, because knockdown by RNAi in another insect study was readily detected after 48 hours (Araujo et al. 2006).

Our study provides the first insight into a possible association between expression of the foraging gene and a foraging state in queens in the eusocial Hymenoptera, which

complements previous studies with workers. We show, for the first time, an association between the foraging gene and queen reproductive status, which supports the 'reproductive groundplan hypothesis' that genes involved in division of labour in workers, such as the foraging gene, are also related to reproduction (Grozinger et al. 2007). Contrary to expectations based on the past studies (Ben-Shahar et al. 2002; Tobback et al. 2011; Rodriguez-Zas et al. 2012), which suggested that *for* expression would be lower in non-foraging queens, we found that *for* expression in both the brain and digestive tract was significantly higher in laying queens, even though laying queens are normally associated with non-foraging behaviour (also supported in this study, see below). The effect found is therefore more consistent with the finding of Kodaira et al. (2009), that foraging *Bombus ignitus* workers had lower *for* expression than non-foragers. However, as in the study of Kodaira et al. (2009), our effect may have been confounded by age (Tobback et al. 2011), because laying queens in our study were on average younger than non-laying queens, and a previous study found decreased expression in older queens (Woodard et al. 2013), although we found no significant effect of age on expression. If real, the negative association of foraging and *for* expression would be in line with the relationship observed in ants and wasps (see *Introduction*). Previous insect microarray studies (i.e. analysing the expression of large numbers of genes) have also revealed nutrition-based changes in expression levels, both in *A. mellifera* (Ament et al. 2010) and *D. melanogaster* (Kent et al. 2009). Our study indicates a relationship between *for* expression and feeding regime but this relationship was opposite in the two tissues studied (fig. 5.3), with ad-libitum fed queens having higher expression in brains and food-restricted queens having higher expression in digestive tracts. Different expression responses to feeding regime by the digestive tract and brain indicates that the foraging gene is involved in at least two separate responses to changes in nutritional state in different tissues, and may be related to both physiology and behaviour, both of which have been shown to respond to nutritional state in honey bees (Ament et al. 2010; Zayed and Robinson 2012). The finding that expression levels in the brain were not related to expression levels in the digestive tract, across all queens, also supports the hypothesis that the foraging gene is associated with multiple functions in the body, because it suggests that *for* expression across the body is not responding to a single stimulus.

Despite the findings above, we found no effects of *for* expression in either the brain or the digestive tract on activity or rhythmicity. This suggests that the *for* gene, although associated with foraging behaviour in bumble bee workers and other insects, is not

always directly linked with locomotor activity or circadian rhythmicity and that, at least in bumble bees, *for* is unlikely to play a central role in these specific behaviours in queens (but could still be related to a foraging state in other ways). Thus, other factors must be important for determining these behaviours, which may include the expression of other genes which are linked with age, reproductive status and/or nutritional status. In agreement with Eban-Rothschild et al. (2011), we found that laying queens had a significantly lower level of circadian rhythmicity in locomotor activity compared to non-laying queens, but this held only for queens which had been food restricted. We found an increase in the overall activity of laying queens relative to non-laying queens and an independent effect of a decline in activity with increasing age, since young non-laying queens in our experiment were more active than older non-laying queens (e.g. amongst ad libitum fed queens, which were non-laying; fig. 5.6c). Thus, surprisingly, a number of our young ad libitum fed non-laying queens tended to be active and arrhythmic (fig. 5.5), a behavioural state associated with non-foraging, despite none of these queens going on to lay eggs in the near future. This further emphasises that there is no simple relationship between locomotory behaviour and reproductive status alone. Only these young aged ad libitum-fed queens in our study showed arrhythmic behaviour amongst non-laying queens, but it was not possible to conclude whether this lack of rhythmicity was due to feeding regime or age. Mean locomotor activity was highly significantly negatively correlated with the probability of having circadian rhythmicity, confirming the same relationship previously found in bumble bee queens, i.e. that confined queens with circadian rhythmicity have lower mean activity than arrhythmic queens. Unsurprisingly, therefore, we found that mean activity was significantly higher in young queens and tended to be higher in laying queens (the same general pattern as arrhythmicity).

Why age effects on locomotor activity in queens should occur independently of laying status is not clear, although it is possible that queen fertility declines with age and so recently emerged queens show a greater preparedness for colony founding (which is linked with both greater activity and arrythmicity); for example, queens of *Bombus impatiens* already have well developed ovaries 6 days after eclosion (Vogt et al. 1994). A causal relationship between feeding regime and foraging behaviour may also be predicted, because a queen with a large amount of stored food resources should have less need to forage, and so ad libitum (i.e. plentiful) resources could act as a stimulus for queens to cease foraging. Indeed, workers in other eusocial insect species have been shown to increase foraging as a response to greater nutritional needs at the

colony and/or individual level (Mailleux et al. 2010; Mayack and Naug 2013), and so it is possible that pre-founding queens also respond in the same way. However, studies designed specifically to compare foraging-associated behaviours between queens of different ages and between queens in different feeding regimes, while controlling for reproductive status, will be necessary to distinguish which of these factors are important in determining the cessation of queen foraging.

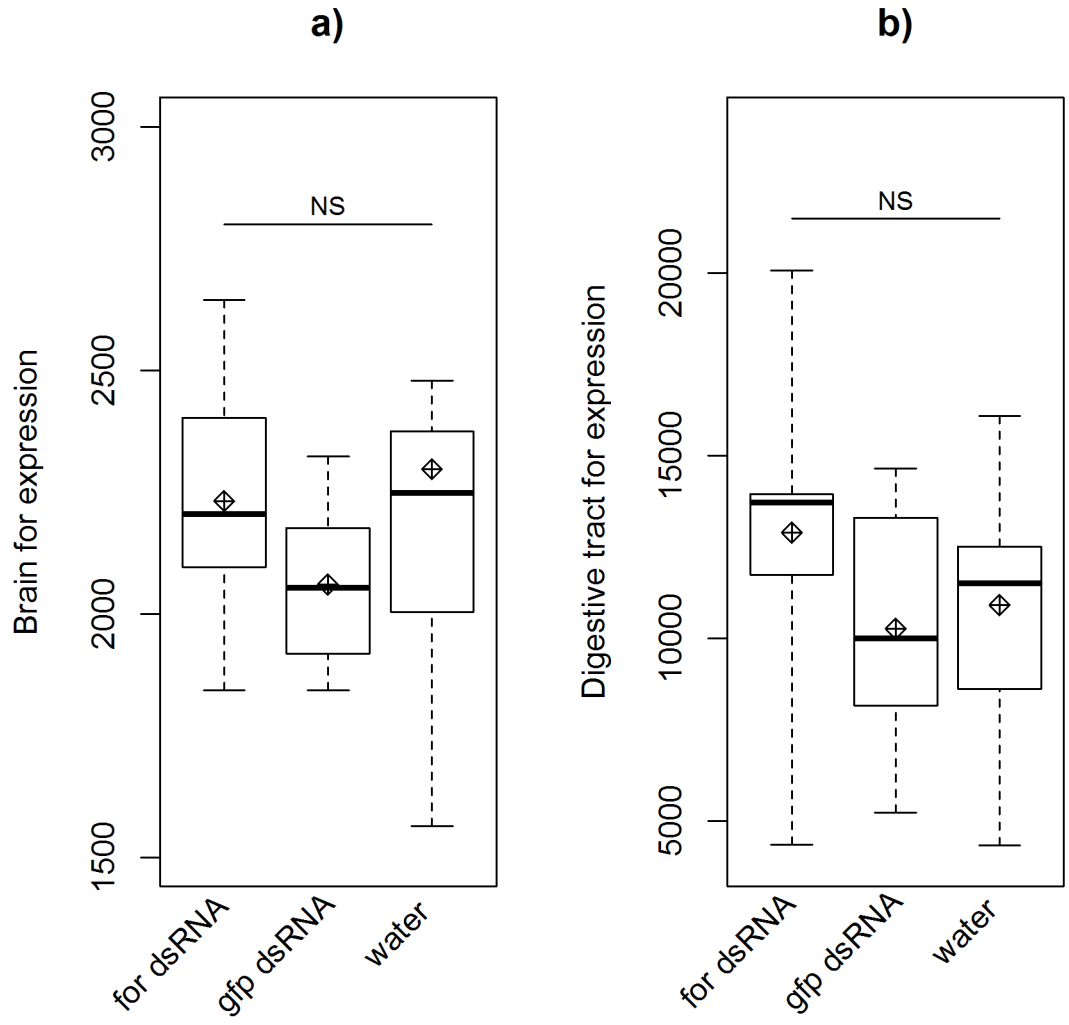

Figure 5.1 *for* expression differences in a) brains and b) digestive tracts of *Bombus terrestris* queens fed with syrup plus one of: *for* dsRNA (n = 18), *gfp* dsRNA (n = 14) or water (n = 14). Expression data, relative to two reference genes, were produced using qRT-PCR (see *Materials & Methods*) and are given in copy number per 2µl of cDNA. The brain expression of one *for* dsRNA queen was not measured, and so is not included in a). Diamonds, means; thick horizontal lines, medians; boxes, interquartile ranges; dotted lines, ranges minus outliers. NS, not significant (ANOVA).

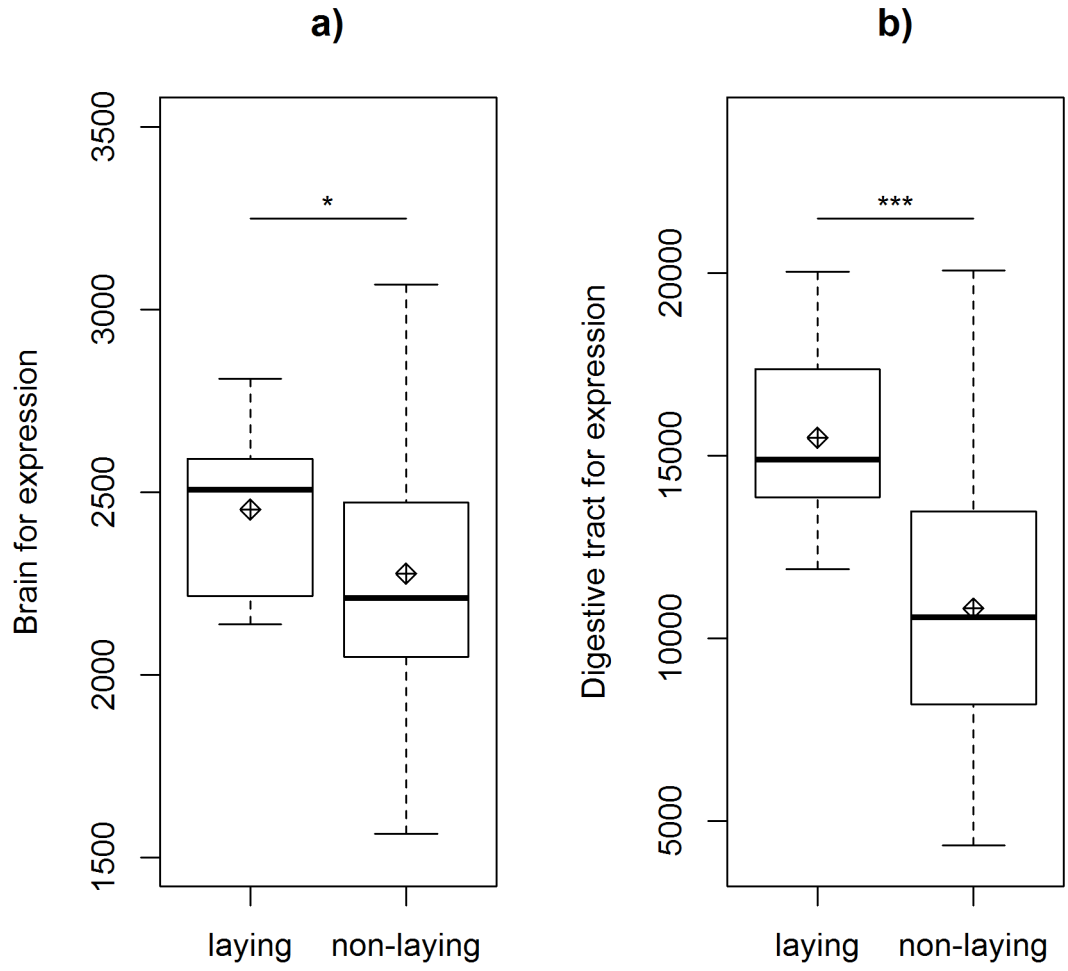

Figure 5.2 *for* expression differences in a) brains and b) digestive tracts of *Bombus terrestris* queens which were either laying (n = 6) or non-laying (n = 66). Expression data, relative to two reference genes, were produced using qRT-PCR (see *Materials & Methods*) and are given in copy number per 2µl of cDNA. The brain expression of one laying queen was not measured, and so is not included in a). Diamonds, means; thick horizontal lines, medians; boxes, interquartile ranges; dotted lines, ranges minus outliers. a) * $p < 0.05$ (ANOVA); b) *** $p < 0.001$ (ANOVA).

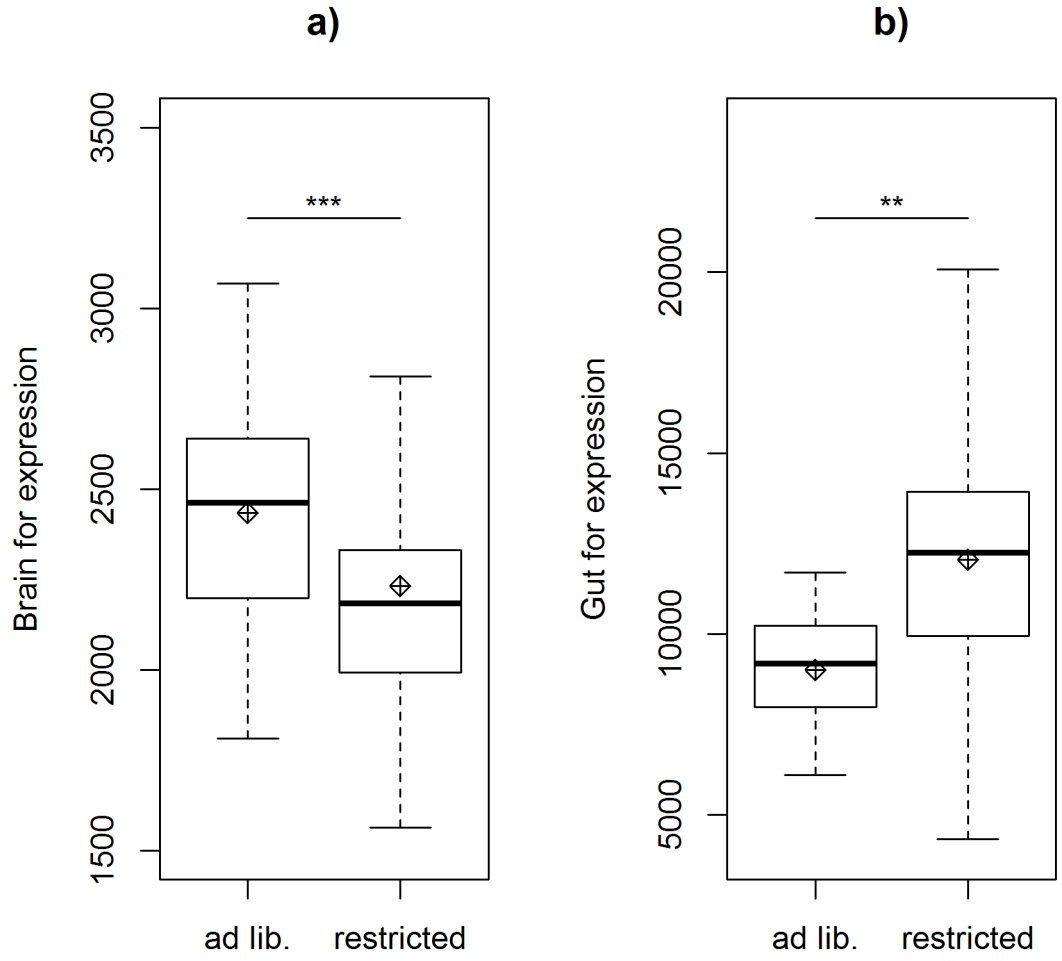

Figure 5.3 *for* expression differences in a) brains and b) digestive tracts of *Bombus terrestris* queens which were given either an ad libitum (n = 20) or restricted (n = 52) feeding regime. Expression data, relative to two reference genes, were produced using qRT-PCR (see *Materials & Methods*) and are given in copy number per 2µl of cDNA. The brain expressions of two food-restricted queens were not measured, and so are not included in a). Diamonds, means; thick horizontal lines, medians; boxes, interquartile ranges; dotted lines, ranges minus outliers. ** $p < 0.01$; *** $p < 0.001$.

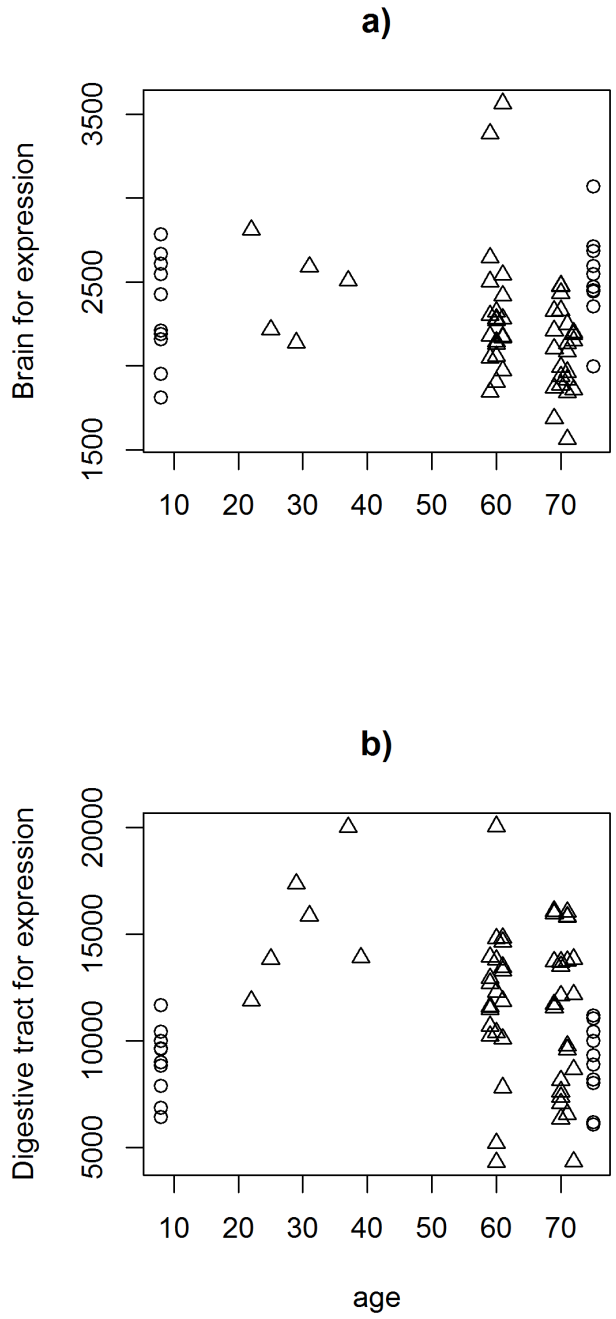

Figure 5.4 *for* expression differences in a) brains and b) digestive tracts of *Bombus terrestris* queens (n=72) sampled at different ages (shown as time after receipt). Expression data, relative to two reference genes, were produced using qRT-PCR (see *Materials & Methods*) and are given in copy number per 2µl of cDNA. Queens subjected to one of two feeding regimes; triangles, restricted (n = 52), or circles ad libitum (n = 20) feeding regime. The brain expressions of two food-restricted queens were not measured, and so are not included in a).

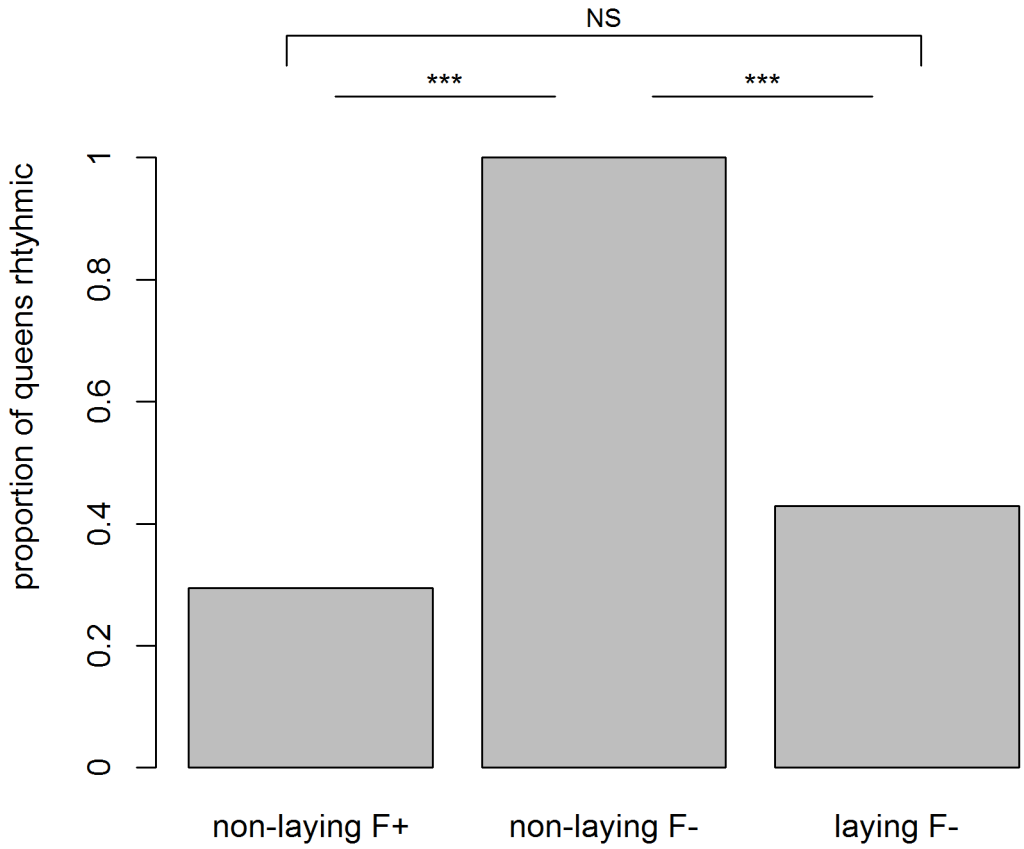

Figure 5.5 Proportion of *Bombus terrestris* queens exhibiting circadian rhythmicity as a function of laying status and feeding regime. Non-laying F+, non-laying queens with ad libitum food (n = 17); non-laying F-, non-laying queens with restricted food (n = 46); laying F-, laying queens with restricted food (n = 6). NS, not significant (chi-squared test); *** $p < 0.001$ (chi-squared test).

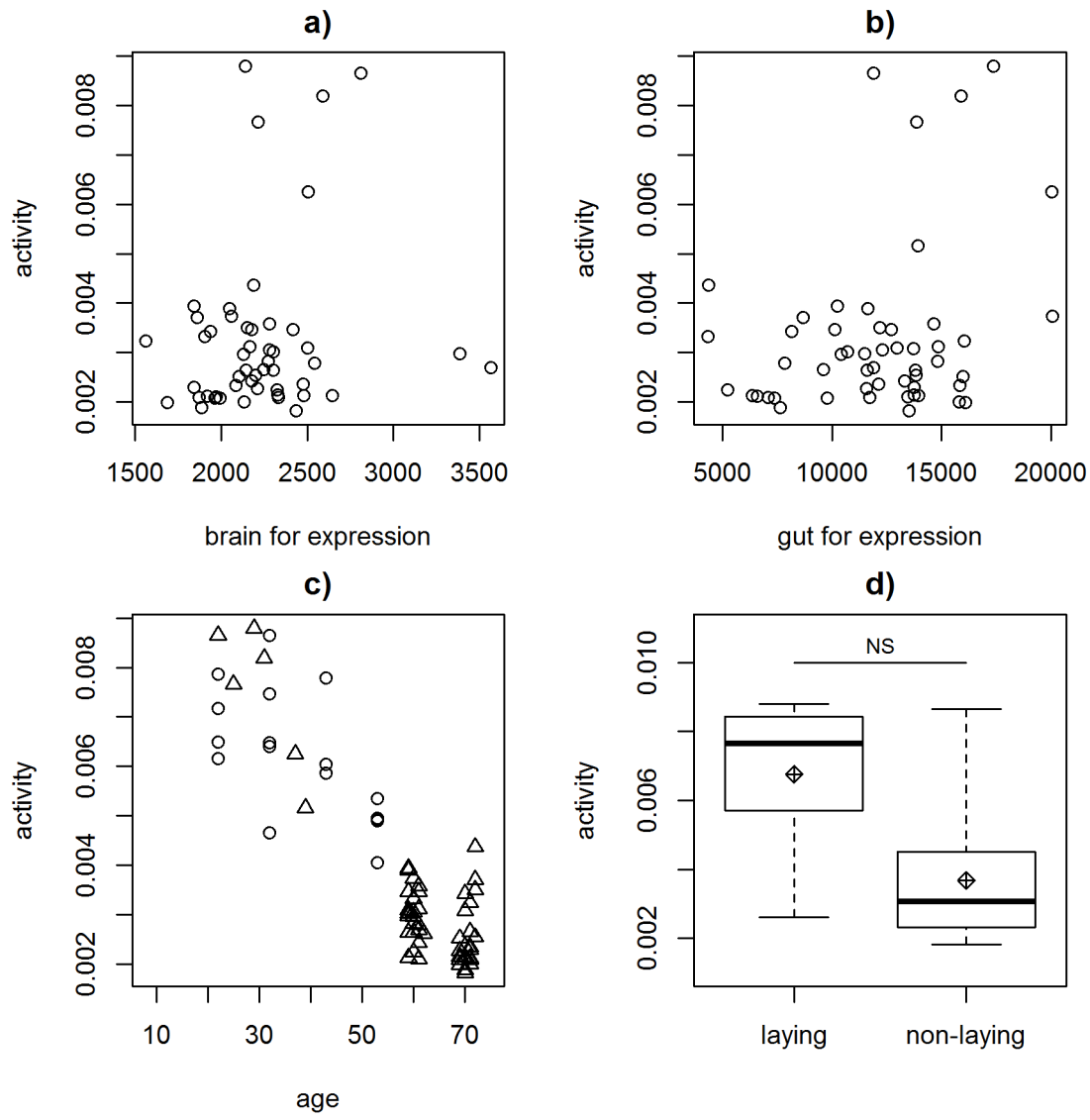

Figure 5.6 Mean activity of *Bombus terrestris* queens in movement (arbitrary units) per second as a function of a) brain *for* expression; b) digestive tract *for* expression; c) age; and d) laying status. Activity measured using movement tracking software (see *Materials & Methods*). For figures a) and b), expression data, relative to two reference genes, were produced using qRT-PCR (see *Materials & Methods*) and are shown as copy number per 2 µl of cDNA. c) age refers to the number of days after the start of experiment that activity tracking (over 7-10 days) ceased; triangles, queens with restricted food regimes; circles, queens with ad libitum food regimes. Total sample sizes 50, 52, 70 and 70 for each subplot respectively. Diamonds, means; thick horizontal lines, medians; boxes, interquartile ranges; dotted lines, ranges. NS = not significant (ANOVA).

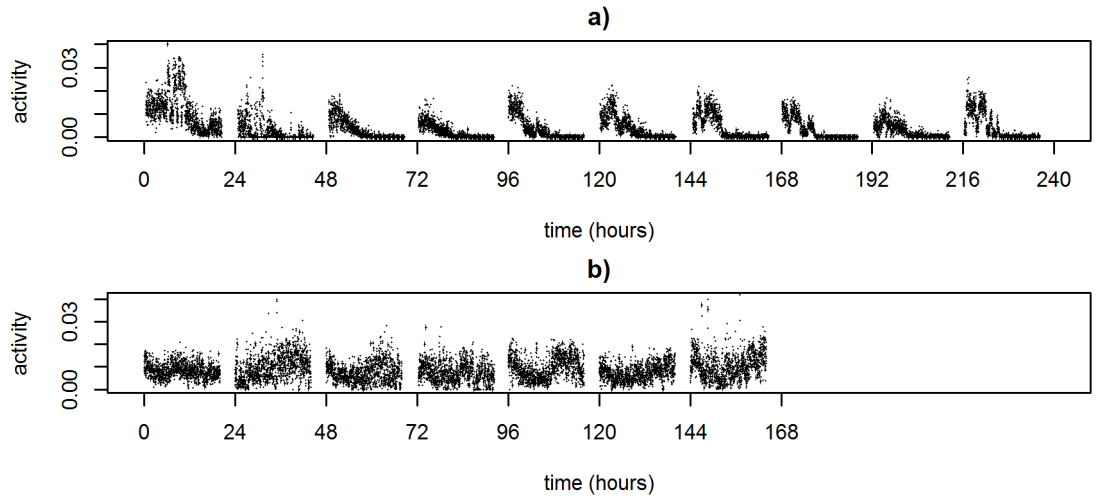

Figure 5.7; Examples of locomotor activity patterns in a) rhythmic and b) arrhythmic queens of *Bombus terrestris.* Locomotor activity (i.e. movement on foot; arbitrary units) of caged queens was recorded using real-time movement tracking software over 7 or 10 day periods for 20 hours per day (see *Materials & Methods*). Gaps in data points show times at which no recording was conducted (four-hour periods between 1300-1345 and 1700-1745 each day). Figure a) shows a non-laying queen with significant circadian rhythmicity, and figure b) shows a laying queen with no circadian rhythmicity.

Table 5.2; PCR and qRT-PCR primers used (see *Materials & Methods*).

Primer Name	Sequence
for forward	GGATCTTCGACCACTGGCTA
for reverse	GCAAAAGATTCTCCGGTTTG
T7 *for* forward	TAATACGACTCACTATAGGGAGAGGATCTTCGACCACTGGCTA
T7 *for* reverse	TAATACGACTCACTATAGGGAGAGCAAAAGATTCTCCGGTTTG
gfp forward	GCCACAAGTTCAGCGTGTCC
gfp reverse	TTCTGCTTGTCGGCCATGAT
T7 *gfp* forward	TAATACGACTCACTATAGGGAGAGCCACAAGTTCAGCGTGTCC
T7 *gfp* reverse	TAATACGACTCACTATAGGGAGATTCTGCTTGTCGGCCATGAT
Reverse transcription universal primer	T(20)VN
ArgK qPCR forward	TGACCCAGCTGGTGAGTTC
ArgK qPCR reverse	TACTGAGCTTCAGTCAGGCAG
ArgK qPCR probe	TGTAAGATGCGGCCGTTCCCTGGAA
for qPCR forward	ATCGATCACACGAAATGCAACT
for qPCR reverse	TGTATTTCGCTAATGCCACCTT
for qPCR probe	CAGGATTATCCCTGCACAGCTT

Chapter 6

General conclusions

The main aim of this thesis was to investigate the social interactions and other mechanisms regulating the timing and control of life history traits in eusocial insects, using the bumble bee *Bombus terrestris* as a model system. The results have advanced our understanding of the evolution of life history and of the eusocial insects, and also provided information useful for the ecological and commercial management of bumble bees. In this chapter, the main findings of the thesis, and the advances in understanding they represent, are outlined ('Main findings'). There follows a more general discussion of the importance of these findings to the above goals ('Importance for social evolution' and 'Importance for ecological and commercial management'). Suggestions for future research in light of the findings presented here are made throughout the chapter.

Main findings

Sexual maturation is an important life history trait at the colony level, which represents the main route to increasing fitness in the eusocial insects. In Chapter 2, I showed that the occurrence of the switch point in *Bombus terrestris*, i.e. the time at which the colony begins male production, is under the endogenous control of the queen because it occurs in the absence of workers. Furthermore, I found no difference in the timing of this event when workers were absent, suggesting that workers are unable to influence when it occurs. For the first time in a eusocial insect, this provides evidence that colony-level sexual production is under direct control of the queen and that workers possess little or no power to determine its occurrence or timing.

Eusocial insects are vulnerable to threats from both inside and outside the colony and have evolved responses to counter these threats. These responses are a key part of the maintenance of eusociality, but how they change throughout colony development in accordance with both proximate and ultimate causes is not well understood. In Chapter 3, I found that *Bombus terrestris* workers do not discriminate between eggs laid by nestmate and non-nestmate workers, even though in nature they may encounter eggs laid by non-nestmate workers acting as intra-specific social parasites.

This demonstrates that *Bombus terrestris*, unlike the handful of other eusocial Hymenoptera species studied in this respect, are almost certainly unable to derive the origin of worker laid-eggs from properties of the eggs themselves. Furthermore, I discovered that the frequency with which eggs are eaten did not differ according to whether or not the colony had passed the competition point, i.e. whether or not natal workers in the host colony had begun to lay their own eggs. This contrasted with the prediction that, if egg-eating is an evolved mechanism to outcompete rival egg-layers, workers or queens should show egg eating only after natal workers begin egg-laying. These results instead give further credence to the possibility that indiscriminate eating of worker eggs could have first evolved as a defence against intra-specific parasites in bumble bees and other eusocial insects.

Understanding how the colony life history of eusocial insects responds to climate change has received little attention, and yet will prove important for the conservation of pollinators. In Chapter 4, I showed that colonies of *Bombus terrestris* reared at either 20^{o}C or 25^{o}C do not greatly vary in colony longevity, or the longevity of queens and workers. This finding suggests that the occurrence of winter activity in *Bombus terrestris* is not due to a plastic response of extending colony or individual persistence under higher temperatures. I also demonstrated that the production of new queens significantly increased with increasing temperature, and that the production of workers and males is also likely to be affected in the same direction. Finally, I show that the timing of peak colony size or first male production was not significantly affected by temperature, which may mean colonies are susceptible to a mismatch in phenology with their food plants under climate change. These experiments are, to my knowledge, the first to test the effects of temperature on colony life history traits such as colony longevity and colony productivity in eusocial insects. The results suggest that temperature is likely to be more important for colony productivity than colony longevity in these systems, and that the longevity of individuals is relatively robust to temperature change, perhaps due to nest thermoregulation.

At a finer scale, the underpinning of colony life history is regulated by molecular and physiological mechanisms within individuals, and yet few studies have investigated these internal processes with respect to colony life history. In Chapter 5, I demonstrated that brain and digestive tract expression of the foraging gene is higher in laying queens of *Bombus terrestris* than in non-laying queens, suggesting that the foraging gene may be implicated in the life history shift to colony foundation. I showed that, despite its association with foraging behaviour in the workers of many eusocial

insect species, foraging gene expression was not always associated with locomotor activity in queens. I also showed that the feeding regime of queens significantly affected expression, with opposite effects in different tissues, suggesting multiple functions of the gene. Finally, I demonstrated that queen age and/or feeding regime affects locomotory behaviour, suggesting these factors might be involved in initiating the shift to a non-foraging state at colony foundation.

Importance for social evolution

Annual species are more constrained with regards to timing than are perennial species. This is because perennial colonies have the potential to develop over a number of years, whereas annual colonies not producing sexuals by the end of the favourable season are unlikely to contribute any genes to future generations. Thus annual species must contribute what they can to sexual production at the right time, whereas perennial colonies have the option to focus on developing the colony for reproduction in future years or seasons (Oster and Wilson 1978; Bourke and Franks 1995). This may be one reason why the experiments reported in Chapters 2 and 4 revealed a relatively inflexible timing of colony life history in *Bombus terrestris*, i.e. 1) that queens do not greatly modify the timing of the switch point in response to changes in the social environment (Chapter 2); and 2) that the timing of first male emergence and peak worker production, as well as colony longevity, do not greatly respond to changes in temperature (Chapter 4). Both cases are consistent with the colony preparing to reproduce at a set time, regardless of environmental conditions, in a limited season; for example, if the queen is not able to successfully produce many workers, or the environmental conditions are suboptimal for mating, it may still be beneficial for the colony (all individuals) to try to produce sexuals at a similar time, because there may not be another chance. This is consistent with the greater relative investment in sexual reproduction seen in annual multicellular organisms, when compared to perennials (e.g. van Kleunen 2007).

The research presented here also emphasises the role of heuristic (rule of thumb) mechanisms in regulating colony life history. For example, in Chapter 2 it was shown that, by not responding to their social environment, queens probably do not use current information from the external environment in determining when to switch to male production (this can be inferred because queens themselves are not exposed to the external environment). Although the external environment may be important for the fine-scale optimal timing of male production, the queen instead relies on an internal

timing mechanism, which under most circumstances should act as a proxy for the external environmental conditions. It is possible that the queen may refine the timing of this event in accordance with other nest conditions such as temperature, but the results from Chapter 4 suggest that this is not case, since we found no significant difference in the timing of first adult male emergence between two nest temperature treatments. In another example of a heuristic mechanism, we found that individuals in a colony do not differ in their response to worker-laid eggs according to colony of origin or according to the host colony's life-history phase. This suggests that the same mechanism is used for both defence against parasites and the suppression of rival egg-laying workers throughout colony life history (Chapter 3).

Heuristic mechanisms are frequently employed in the behaviour of individual organisms, and represent a route to achieving generally adaptive responses by the use of a few simple rules (Goodie et al. 1999). However, the simplicity of heuristic rules may not always produce optimal results, suggesting possible constraints in evolving more highly attuned responses. For example, in the case of switch point occurrence in *B. terrestris*, queen control without worker influence may be retained because 1) it is difficult to evolve the mechanism of a more finely tuned integrated control system, and/or 2) retaining this internal mechanism is, ultimately, more beneficial to the queen because it allows her to deny power to workers, which may otherwise skew the timing or occurrence of the switch point in their own interests. If the second reason is correct, simple queen-internal heuristic mechanisms may be lost in more advanced eusocial societies where there is less actual conflict between workers and the queen (see Ratnieks et al. 2006a; Bourke 2011). In other words, individuals in social systems with a greater alignment of interest (Ratnieks and Reeve 1992; Wenseleers and Ratnieks 2004; Bourke 2011), or less power to defect (Ratnieks and Reeve 1992; Beekman et al. 2003; Helantera and Ratnieks 2009; Ratnieks and Helantera 2009), may be more willing to utilise complex information from other individuals, because there is less incentive for individuals to manipulate colony life history towards individual interests. If this is true, colony members in these species would be predicted to integrate information from a larger number of individuals in order to make life history decisions, with different individuals specialized to collect information from different sources, i.e. to have a greater functional integration (Anderson and McShea 2001). Since an understanding of the regulation of colony life history is only available for a minority of species, whether this is true remains to be seen. However, honey bee colonies, which possess low levels of defection (due to policing; Wenseleers and Ratnieks 2006b),

show a high level of complexity in determining the life history decision of where to go in colony swarming, which integrates information from multiple individuals (Seeley 2010). The greatest test of this hypothesis would be to investigate the regulation of life history in clonal societies, such as parthenogenetic ants (e.g. Teseo et al. 2013) and eusocial gall forming aphids (e.g. Uematsu et al. 2010). Since these systems have a high alignment of interest (as r≈1 between nestmates), they would be predicted to exhibit a high degree of functional integration in colony life history regulation. Indeed, the regulation of life history in multicellular organisms (in which r = 1 between cells in different tissues) may occur by more complex, multifaceted mechanisms, which integrate and respond to information from multiple sources (which must be collected from multiple tissues) (e.g. De Block and Stoks 2003; Elekonich and Roberts 2005; Flatt et al. 2005; Gomez-Mestre et al. 2008), allowing life history to plastically respond to the environment in an adaptive fashion (Nylin and Gotthard 1998; West-Eberhard 2003).

Finally, Chapter 5 provided the first study into the genetic control of a colony life history event in a eusocial insect. Since the shift from a solitary to eusocial state is likely to be regulated by genes in only a single individual (the foundress queen) that, as demonstrated in Chapter 2, can nonetheless influence colony life history, this shift was an ideal starting point for research. Here I showed that the foraging gene may be implicated in colony foundation, since it is more highly expressed in laying queens. By identifying a possible effect of nutrition and age on locomotor activity, I also suggested additional physiological processes that may be involved in regulating this event. Nevertheless, a fuller understanding of the genetic control of colony life history events will require a combination of candidate gene manipulations, which can help to establish causality (Ben-Shahar et al. 2002), and whole genome approaches, which can help to identify the gene networks involved (Rodriguez-Zas et al. 2012; Zayed and Robinson 2012). The genetic control of complex life history events involving interactions between many individuals will be particularly difficult to analyse, because it must involve genes interacting with genes in other individuals, i.e. it must involve extended phenotypes (Dawkins 1982). Beginning to understand this genetic control will not only help to bridge the gap between genotype and social phenotype by understanding how colony life history is mechanistically regulated, but will also shed light on the evolution of eusociality by revealing which genes and molecular pathways were the antecedents of eusocial traits (Sumner 2006; Grozinger et al. 2007; Hunt et al. 2007).

Importance for ecological and commercial management

In order to manage social insect populations in the wild, or in colonies reared by humans, it is important to understand how the colony growth schedule responds to changes in the external environment. The robustness in the timing of colony sexual maturation, peak worker production and colony death found in Chapters 2 and 4 suggests that it may be difficult to alter the growth schedule of annual social insect colonies, at least in *Bombus terrestris*. However, Chapter 4 highlighted the importance of temperature for colony productivity, in particular the production of new queens, which has ramifications in the conservation of bumble bee populations. For example, if summer temperatures increase, this is may alter the population sex ratio in favour of new queens (over males).

The presence of winter active bumble bees may be of concern from a conservation perspective, particularly if the foundation of winter colonies is occurring, because colonies developing in winter may be incapable of producing sexuals (as appears to be the case in southern Britain; Stelzer et al. 2010) and so the shift to year-round activity could be maladaptive. However, more work will be necessary to identify which mechanisms drive queens to found colonies without diapause in nature (Chapter 4).

Changes in colony-level productivity and sex-ratio (above) are also important for efficiency in agricultural bumble bee rearing, which is used worldwide for the production of food crops. We show that the most appropriate way to alter the timing of colony sexual maturation, which may be important for increasing efficiency, is likely to be treatment of the queen, such as alteration of the diapause regime (Beekman and Van Stratum 2000; Gosterit and Gurel 2009). This is because manipulations of workers are unlikely to affect the timing of male production (Chapter 2). Chapter 5 suggests molecular and physiological pathways which may be implicated in colony foundation, such as the foraging gene and responses to nutrition and age. If these pathways can be better understood, they may suggest new ways in which colony foundation can begin more efficiently, e.g. alternative external factors that can trigger the onset of colony foundation faster than current practices.

Glossary of key terms

Definitions of technical terms frequently used in this thesis:

cDNA –Complementary DNA; that is, DNA formed from mRNA via reverse transcription.

Circadian rhythmicity –The state of having cycles in activity (e.g. behavioural locomotor activity) which have periods of approximately one day (usually 20-28 hours).

Competition Point –In annual bumble bees, the time at which some workers begin laying eggs. It is generally also accompanied by increases in worker-worker and worker-queen aggression, as well as egg eating behaviour.

Diapause –a period of inactivity and reduced metabolism which many temperate insects exhibit during winter (in which case it is also referred to as hibernation), but which may also occur at other times.

Eusociality –the condition of having the cohabitation of multiple conspecifics, overlapping in generation, and exhibiting both cooperative brood care and a reproductive division of labour (i.e. some individuals reproduce more than others) within the group. Considered a level of social organisation.

Hymenoptera –The insect order that includes ants, bees and wasps.

Microsatellite –A non-coding region of DNA which is comprised of short repeating sections of 2-6 nucleotides. Due to the error rate associated with reproduction of these loci, they are highly variable (polymorphic) in repeat number within species.

Queen –In eusocial insects, the name usually given to females capable of high levels of reproduction. They are often morphologically distinct from other classes of females (e.g. workers), and are responsible for colony foundation in the majority of species.

Relatedness (r) – The probability that the two focal individuals will share any given allele.

Switch Point –In annual bumble bees, the time at which the queen shifts between producing diploid eggs, yielding males, and haploid eggs, yielding females. Strictly defined as the first haploid egg laid.

Worker –In eusocial insects, the name usually given to individuals with a low reproductive capacity and which are incapable of mating. In the eusocial Hymenoptera, workers are exclusively female.

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