Sociogenetics and behavioural ecology of the Tree Bumblebee (*Bombus hypnorum*)

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Thesis abstract

Understanding the causes of ecological success in pollinators and the basis of social evolution across all organisms represent goals of broad interest to ecologists and evolutionary biologists. Therefore, the aim of this thesis was to advance our knowledge of each of these topics through investigating the sociogenetics and behavioural ecology of the range-expanding bumblebee, *Bombus hypnorum*. Genetic analyses using microsatellite markers to quantify neutral genetic variation and levels of diploid male production revealed that the UK *B. hypnorum* population did not undergo a genetic bottleneck upon its 2001 colonisation of the UK, suggesting that this population does not represent, as previously suggested, an example of the genetic paradox of invasion (ecological success despite low genetic diversity). Analyses of host-parasite interactions between *B. hypnorum* and the generalist nematode parasite *Sphaerularia bombi* revealed that *B. hypnorum* exhibits partial resistance to the castrating effects of *S. bombi* observed in other bumblebees and may represent a 'dead-end' host for *S. bombi*. Such resistance could facilitate *B. hypnorum*'s ecological success and could benefit long-established UK bumblebee species through parasite dilution. Characterisation of the colony demography of *B. hypnorum* revealed that queen longevity was positively associated with lifetime reproductive success and that *B. hypnorum* exhibits proportionately higher levels of new queen production than bumblebees with stable population trends, which might also contribute to the UK population's rapid growth. Finally, behavioural and genetic analyses of *B. hypnorum* colonies revealed that queen-worker conflict over male parentage is resolved almost exclusively by queen policing, demonstrating the diversity of conflict resolution strategies across eusocial insects. Overall, these findings increase our understanding of ecological success in a key group of pollinating insects and of the evolution of conflict resolution in eusocial societies.

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Author contributions

Chapter 2 | No severe genetic bottleneck in a rapidly range-expanding bumblebee pollinator

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David Richardson, Claire Carvell, Martin Taylor, and Andrew Bourke conceived and supervised the study. Ryan Brock, Liam Crowther, DR, CC, MT, and AB designed the study. RB and LC conducted the field and laboratory work and analyses. Specifically, LC carried out the worker sampling, worker genotyping, and bottleneck analysis, while RB carried out the male sampling, male genotyping, and diploid male analysis. DR, David Wright, and MT provided laboratory and genetic advice. AB produced the model to estimate allelic diversity at the sex-determining locus. RB and AB wrote the manuscript, with input from LC. Overall RB contribution: 45%.

Chapter 3 | Host-parasite interactions between the Tree Bumblebee (*Bombus hypnorum*) and a generalist bumblebee nematode parasite (*Sphaerularia bombi*)

Ryan Brock, Mark Brown, and Andrew Bourke conceived and designed the study. RB and Agnes Rutter conducted the field and laboratory work. Specifically, RB collected queens, reared colonies, carried out parasite screening, and gathered colony demography data for the 2018 colony cohort, and was assisted by AR for the 2019 colony cohort. Further, RB and AR performed queen dissections, while RB performed haemocoel and gut screening of *S. bombi*-infected queens and the photography and measurement of *S. bombi* uteri. RB analysed the data. RB wrote the manuscript, with input from AB. Overall RB contribution: 80%.

Chapter 4 | Colony demography and queen lifetime reproductive success in the Tree Bumblebee (*Bombus hypnorum*)

Ryan Brock and Andrew Bourke conceived and designed the study. RB conducted the field and laboratory work, with assistance from Agnes Rutter. Specifically, RB collected queens, reared colonies, and gathered colony demography data for the 2017 and 2018 colony cohorts, and was assisted by AR for the 2019 colony cohort. RB analysed the data. RB wrote the manuscript, with input from AB. Overall RB contribution: 90%.

Chapter 5 | Queen policing limits worker reproduction in the Tree Bumblebee (*Bombus hypnorum*)

Ryan Brock and Andrew Bourke conceived and designed the study. RB conducted the field and laboratory work, with assistance from Agnes Rutter and Moira Muncy. Specifically, RB collected queens, reared colonies, and gathered colony demography data for the 2018 colony cohort, and was assisted by AR for the 2019 colony cohort. Further, RB and MM measured the dry weights of workers, gynes, and males from each colony. Finally, RB undertook all colony recording and observation, viewing and analysis of the digital films, and microsatellite genotyping of colony queens, workers, and males. RB analysed the data. RB wrote the manuscript, with input from AB. Overall RB contribution: 90%.

Chapter 1

General introduction

A Tree Bumblebee (*Bombus hypnorum*) colony reared from a field-collected queen (top left).

CHAPTER 1

General introduction

1.1. The evolution of eusociality

Eusociality represents an advanced form of social organisation characterised by individuals being divided into reproductive and non-reproductive (helper) castes, belonging to two or more overlapping generations, and exhibiting co-operative offspring care (Crespi & Yanega, 1995; Boomsma & Gawne, 2018). Such a system creates a reproductive division of labour between colony members, with reproductive individuals responsible for most of the offspring produced and helpers responsible for tasks that ensure colony function, such as offspring care, foraging, and colony defence (Crespi & Yanega, 1995; Bourke, 2011a). The reproductive success of eusocial societies is therefore based on mutual dependence between the reproductive and helper castes, characteristic of a major evolutionary transition from solitary individuals to colonial ‘superorganisms’ (Szathmáry & Smith, 1995; Bourke, 2011a; West *et al.*, 2015; Boomsma & Gawne, 2018).

Eusociality represents a striking example of convergent evolution, having independently evolved more than 20 times across a diverse range of organisms (Bourke, 2011a), including mole rats (Jarvis & Bennett, 1993), shrimps (Duffy, 1996; Chak *et al.*, 2017), termites (Thorne, 1997), aphids (Stern & Foster, 1996), thrips (Crespi, 1992), beetles (Smith *et al.*, 2018) and flukes (Hechinger *et al.*, 2011). More recently, eusociality has been suggested in the staghorn fern *Platycerium bifurcatum*, representing the first and only origin of eusociality in plants reported to date (Burns *et al.*, 2021). However, eusociality is most prevalent in the aculeate Hymenoptera (ants, bees, and stinging wasps) where it is exhibited across almost 17,000 species, having independently evolved once in the common ancestor of all ants, three times in the wasps, and four times in the bees (Gibbs *et al.*, 2012; Rehan *et al.*, 2012; Branstetter *et al.*, 2017; Peters *et al.*, 2017). Generally, eusocial hymenopteran colonies consist of one or few reproductive females, the queen(s), and her/their non-reproductive female offspring, the workers (Hölldobler & Wilson, 2009). Hence, eusocial Hymenopteran colonies are composed entirely of females, with males solely produced for mating (Hölldobler & Wilson, 2009). The adaptability afforded by eusociality (e.g. Wilson, 1993; Straub *et al.*, 2015) has led to tremendous ecological success, with eusocial hymenopterans abundant across all continents except Antarctica, as both native and invasive species (Moller, 1996; Hölldobler & Wilson, 2009).

Given that helpers forgo reproduction (and hence their own direct fitness) to assist the reproductive castes, eusociality represents an extreme example of altruism (Hamilton, 1963, 1964a,b). Altruism is best explained by inclusive fitness (or kin selection) theory, where selection favours genes responsible for altruistic behaviour when the benefits to a related recipient outweigh the costs to the actor, thereby increasing the actor's inclusive fitness (direct plus indirect fitness) and propagating the spread of altruistic genes through a population (Hamilton, 1963, 1964a,b). This can be mathematically formalised using Hamilton's rule:

$$rb - c > 0 \qquad \textbf{Equation 1.1}$$

where r represents relatedness (defined as the regression coefficient of shared alleles) between the interacting individuals, b represents the direct fitness benefit to the recipient, and c represents the direct fitness cost to the actor (Hamilton, 1964a,b; Queller, 1992; Grafen, 2006). Eusociality can therefore evolve due to helpers gaining indirect fitness through assisting the reproductive efforts of closely-related individuals, leading to high inclusive fitness gains (Hamilton, 1964a,b). Accordingly, previous research has demonstrated the importance of genetic relatedness (covering the r term in Hamilton's rule), alongside fitness gains and ecological constraints (covering the b and c terms in Hamilton's rule) during the evolution of eusociality (e.g. Chapman *et al.*, 2000; Cant & Field, 2001; Hughes *et al.*, 2008; Leadbeater *et* *al.*, 2011; Ross *et al.*, 2013; Avila & Fromhage, 2015; Kennedy *et al.*, 2018; Field & Toyoizumi, 2020). Nonetheless, the relevance of inclusive fitness theory in explaining altruism, and subsequently eusocial evolution, has attracted criticism (e.g. Wilson & Hölldobler, 2005; Nowak *et al.*, 2010, 2017; Allen *et al.*, 2013; Nowak & Allen, 2015; Olejarz *et al.*, 2015; Allen & Nowak, 2016), with critiques aimed at the simplistic nature of Hamilton's rule and the limited predictive power of inclusive fitness.

A review of the inclusive fitness controversy is beyond the scope of this thesis but examples can be found in Bourke (2011b), Marshall (2016), Queller (2016), Birch (2017), and Levin & Grafen (2019). One major insight from these reviews is that the proposed evolutionary dynamics-based alternatives to inclusive fitness models require overly strict assumptions that would no longer provide universal insights into social evolution. Further, such models fail to yield novel insights into social evolution. For instance, re-analyses of the evolutionary dynamics models suggested by Nowak *et al.* (2010) and Olejarz *et al.* (2015) concluded that relatedness still played an important role in the evolution of eusociality (Liao *et al.*, 2015; Davies & Gardner, 2018). Similarly, a recent analysis showed that the assumptions of 43 models predicting altruistic evolution through means besides relatedness were, in fact, equivalent to assuming relatedness, leading to the 'serial rediscovery' of the key role of relatedness when analysing the evolution of altruism (Kay *et al.*, 2020). Finally, inclusive fitness theory predictions have provided tremendous insight into the co-operation and conflict observed across multiple levels of biological organisation (Trivers, 1974; Queller & Strassmann, 1998; Queller, 2003; Ratnieks *et al.*, 2006; Bourke, 2015), leading to an increased understanding of the major evolutionary transitions (Szathmáry & Smith, 1995; Bourke, 2011a, 2019; West *et al.*, 2015). Hence, inclusive fitness represents an important unifying framework for the study of social evolution (Birch, 2017; Levin & Grafen, 2019), with the theory continuing to guide novel extensions (Kobayashi *et al.*, 2013; Kennedy *et al.*, 2018; Kennedy & Radford, 2021), syntheses (West *et al.*, 2015; Korb &

Heinze, 2016; Bourke, 2019), and empirical tests (Loope, 2015; Almond *et al.*, 2019; Kennedy *et al.*, 2021; Lengronne *et al.*, 2021).

1.2. The evolution, ecology, and importance of bumblebees

The Apidae family contains most of the obligately eusocial bee species, including the honeybees (tribe: Apini), bumblebees (tribe: Bombini), and stingless bees (tribe: Meliponini). Alongside the orchid bees (tribe: Euglossini), that exhibit a range of social states from solitary to simple eusociality (e.g. Andrade *et al.*, 2016; Freiria *et al.*, 2017; Saleh & Ramírez, 2019), these four monophyletic tribes form a monophyletic group within the Apidae, known as the corbiculate bees (Cardinal *et al.*, 2010; Cardinal & Danforth, 2011; Hedtke *et al.*, 2013; Romiguier *et al.*, 2016; Bossert *et al.*, 2017; Branstetter *et al.*, 2017), so called due to the presence of pollen collection structures on the hind legs called corbiculae. Despite historical controversy about the origins of eusociality within the Apidae (Danforth *et al.*, 2013), current genomic analyses confirm a single origin of obligate eusociality in the corbiculate bees, with honeybees, bumblebees, and stingless bees sharing a common ancestor with simple eusocial organisation (Romiguier *et al.*, 2016; Bossert *et al.*, 2017; Branstetter *et al.*, 2017). Ultimately, the social diversity observed within the Apidae (from solitary to eusocial organisation) means they represent an excellent group in which to study eusocial evolution (Kocher & Paxton, 2014; Rehan & Toth, 2015; Shell *et al.*, 2021).

Having diverged from their sister group, the stingless bees, around 70 million years ago (Cardinal & Danforth, 2011), bumblebees (*Bombus* spp.) comprise approximately 250 extant species with eusocial or socially parasitic life-histories, distributed across the Americas, Eurasia and northern Africa (Williams *et al.*, 2008; Goulson, 2009). Unlike honeybees and stingless bees, which exhibit the complex eusocial traits of queen-worker caste dimorphism, large colony sizes comprising thousands of workers, worker sterility and swarm-founding (Michener, 1969; Grüter, 2020), bumblebees exhibit traits associated with both simple and complex eusocial societies (Bourke, 2011a). For example, bumblebees exhibit pre-imaginal, size-based morphological queen-worker differentiation (Amsalem *et al.*, 2015), relatively small colony sizes comprising tens to hundreds of workers, and workers that, while unable to mate, exhibit high reproductive potential via the production of haploid males (Bourke, 1988; Zanette *et al.*, 2012). Hence, bumblebees can be considered as intermediately eusocial (Harrison *et al.*, 2015).

Across temperate zones, bumblebees generally exhibit annual colony cycles (**Figure 1.1**) that can be separated into solitary and social phases. The solitary phase begins with mating between queens and males (**Figure 1.1**), with bumblebee queens known to be mostly monandrous, although a low level of polyandry has been recorded in some species (Schmid-Hempel & Schmid-Hempel, 2000; Paxton *et al.*, 2001; Brown *et al.*, 2002; Cnaani *et al.*, 2002; Huth-Schwarz *et al.*, 2011; Owen & Whidden, 2013; Crowther *et al.*, 2019). Following the mating period, males die, and mated queens enter hibernation (**Figure 1.1**), with queen diapause lasting 3-9 months (Alford, 1969, 2011). However, diapause may not always be necessary for bumblebee queens, as evidenced by perenniality in some tropical bumblebee species (Cameron & Jost, 1998) and the suggestion of bivoltinism (two colony cycles per year) in some temperate bumblebee species (Edwards & Jenner, 2005; Stelzer *et al.*, 2010; Skyrm *et al.*, 2012). Following emergence from hibernation, queens initiate colonies independently by finding a suitable nest site, laying the first brood of worker-destined eggs, and foraging for nectar and pollen with which to provision the developing workers (Goulson, 2009; Alford, 2011; **Figure 1.1**).

The social phase begins with the eclosion (emergence of adults from pupae) of the first workers, which take over foraging and brood care responsibilities, enabling the queen to focus exclusively on egg production (Duchateau & Velthuis, 1988; Goulson, 2009; Alford, 2011). Colony size increases as the queen produces more workers during the growth phase, before the queen transitions to producing gynes (virgin queens) and males during the reproductive phase (Duchateau & Velthuis, 1988; Beekman *et al.*, 1998; **Figure 1.1**). The onset of sexual production is closely followed by queen-worker conflict over male production, known as the 'competition point' (Duchateau & Velthuis, 1988; Bloch, 1999). At this point, due to haplodiploid sex determination, workers may begin laying haploid, male-destined eggs (Duchateau & Velthuis, 1988; Zanette *et al.*, 2012) to which they are more highly related than to queen-produced males (i.e. their brothers; Bourke, 1988; Hammond & Keller, 2004). In extreme cases, such conflict may result in the workers killing the mother queen to gain control over male production ('worker matricide'; Bourke, 1994). Following the competition point and queen death, workers may continue to produce some males (e.g. Duchateau & Velthuis, 1988; Lopez-Vaamonde *et al.*, 2009; Almond *et al.*, 2019) before the colony gradually declines and dies, bringing the colony cycle to an end (Goulson, 2009; Alford, 2011). Upon sexual maturation, the newly produced gynes and males leave the colony to mate (**Figure 1.1**), typically in outbred matings, beginning the colony cycle again.

The eusocial Hymenoptera provide essential ecosystem services, defined as the provision of goods that directly or indirectly support the quality of human life (MEA, 2005), across both natural and agricultural habitats (Losey & Vaughan, 2006; Del Toro *et al.*, 2012; Elizalde *et al.*, 2020; Brock *et al.*, 2021a). In particular, bumblebees represent key pollinators of many economically important crops and wild plants worldwide (Burkle *et al.*, 2013; Garibaldi *et al.*, 2013; Garratt *et al.*, 2014; Reilly *et al.*, 2020). For instance, the generalist nature of bumblebee pollination sustains diverse plant communities through the visitation of both rare and abundant plant species in natural ecosystems (Brosi & Briggs, 2013; Burkle *et al.*, 2013; Brosi *et al.*, 2017). Further, in agricultural ecosystems, bumblebee pollination services produce higher yields across various economically important crops (e.g. Dogterom *et al.*, 1998; Serrano & Guerra-Sanz, 2006; Dimou *et al.*, 2008), leading to the commercialisation of bumblebees for greenhouse use (Velthuis

& van Doorn, 2006). Hence, alongside other pollinating insects, pollination services provided by bumblebees support global food security (Losey & Vaughan, 2006; Klein *et al.*, 2007; Gallai *et al.*, 2009), with a recent study having estimated that the loss of such insect pollinating services would cost 1-2% of global GDP (Lippert *et al.*, 2021).

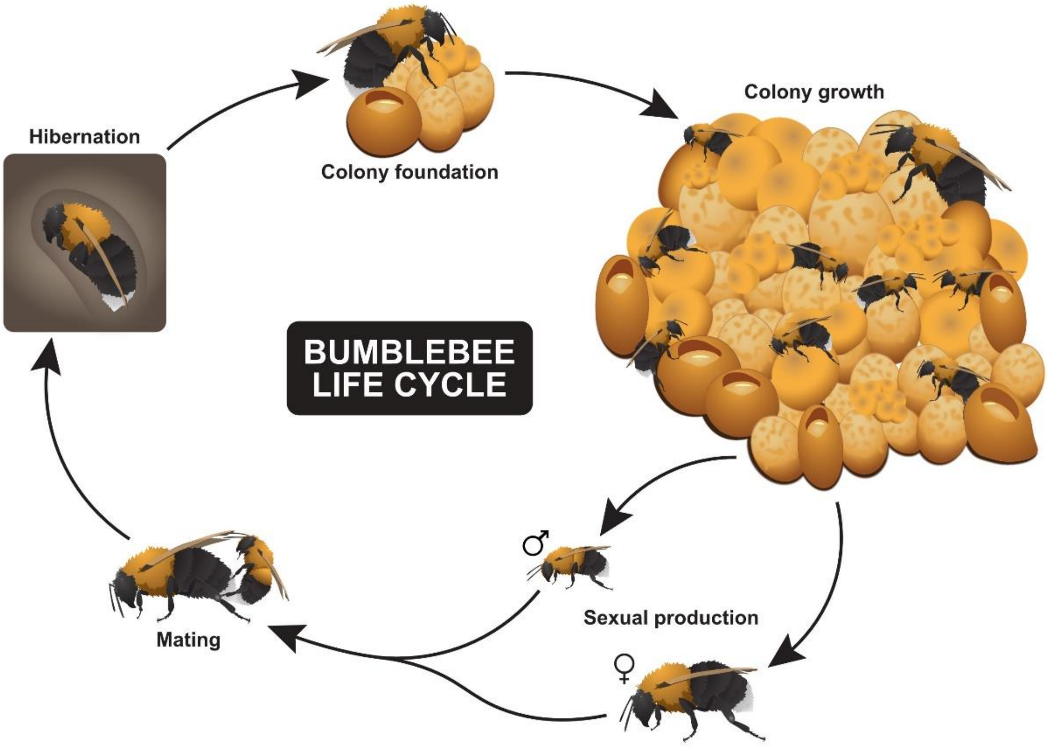

Figure 1.1. The typical colony cycle of temperate bumblebee (genus: *Bombus*) species. The colony cycle begins with foundress queens emerging from hibernation to forage and begin nest-searching. Once a suitable nest site is located, the queen will begin to lay and rear fertilised, diploid eggs (that develop into female workers). Once the queen's first brood has eclosed, workers take over tasks essential for colony function, such as foraging and brood care, leaving the queen to focus on egg production. The colony continues to develop in this manner until the queen begins to produce sexuals (males and gynes). Once developed, sexuals leave their natal nest (which dies soon afterwards) to mate with foreign partners. Males die after mating, leaving inseminated gynes to hibernate and restart the colony cycle.

Worryingly, these pollination roles are threatened by widespread declines in bumblebee populations (Williams *et al.*, 2009; Cameron *et al.*, 2011; Dupont *et al.*, 2011; Arbetman *et al.*, 2017), with such declines thought to be driven by multiple interacting factors (Goulson *et al.*, 2008, 2015; Potts *et al.*, 2010; Cameron & Sadd, 2020) including habitat loss (Vray *et al.*, 2019; Hemberger *et al.*, 2021), pathogen infection (Arbetman *et al.*, 2017), pesticide usage (McArt *et al.*, 2017);, and climate change (Kerr *et al.*, 2015; Soroye *et al.*, 2020). However, at regional scales, some bumblebee species are expanding in both abundance and range as either human-introduced invasive species (Semmens *et al.*, 1993; Matsumura *et al.*, 2003; Schmid-Hempel *et al.*, 2013) or through natural range expansion processes (Šima & Smeta, 2012; Martinet *et al.*, 2015; Biella *et al.*, 2020; Rollin *et al.*, 2020). Accordingly, there is special interest in the genetic, ecological, and evolutionary factors that underlie successful range-expansion processes in bumblebees (Allen *et al.*, 2007; Schmid-Hempel *et al.*, 2007; Crowther *et al.*, 2014, 2019; Jones & Brown, 2014; Biella *et al.*, 2020; Huml *et al.*, 2021), along with the potential impacts range-expanding bumblebees may have in their new ranges (Kingston & McQuillan, 1998; Inoue *et al.*, 2008; Madjidian *et al.*, 2008; Kondo *et al.*, 2009; Arbetman *et al.*, 2013; Morales *et al.*, 2013).

1.3. The Tree Bumblebee (*Bombus hypnorum*): a range-expanding bumblebee pollinator

The Tree Bumblebee (*B. hypnorum*) represents one of the most widespread bumblebee species across Eurasia, with a historical distribution ranging longitudinally from western mainland Europe to Japan and latitudinally from Arctic Russia to Nepal (Goulson & Williams, 2001; Rasmont *et al.*, 2015). Across its range, *B. hypnorum* is associated with a broad range of biomes, from boreal forest in the north to montane forests in the south, but is absent from Eurasian steppe and Mediterranean scrub habitats (Goulson & Williams, 2001; Rasmont *et al.*, 2015). *B. hypnorum* is also highly associated with urban environments (Loken, 1973; Crowther *et al.*, 2014), which is likely to be due to human habitations suiting its preference for nesting in arboreal and other above-ground cavities, with nests commonly being located in bird boxes and roof cavities (Prŷs-Jones, 2014, 2019; Goulson *et al.*, 2018).

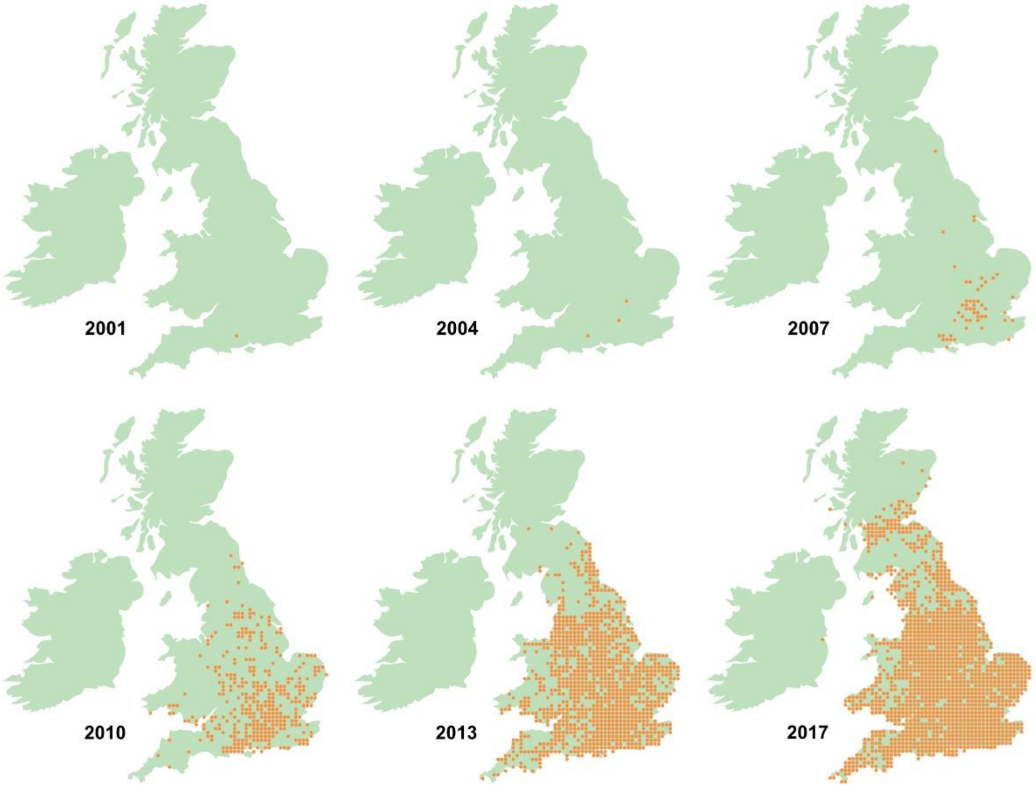

Figure 1.2. The arrival and rapid range-expansion across the British Isles by the Tree Bumblebee (*Bombus hypnorum*) from 2001-2017, following its initial recording in Wiltshire, southern England (Goulson & Williams, 2001). Each orange dot represents confirmed occupancy by *B. hypnorum* across a 10 km square. Maps recreated from the *B. hypnorum* mapping project (BWARS, 2019).

Limited data have demonstrated increases in *B. hypnorum* abundance across Germany and Belgium during the 20th century (Wagner, 1937; Rasmont, 1989), and its colonisation of north-western France during the 1980s (Rasmont *et al.*, 2015). More recently, *B. hypnorum* has exhibited range expansions across the UK and Iceland from 2001 and 2008, respectively (Goulson & Williams, 2001; Prŷs-Jones *et al.*, 2016). Following its arrival in the UK, *B. hypnorum* has rapidly increased in range and abundance to become one of the most common and widespread UK bumblebee species (Crowther *et al.*, 2014, 2019; Jones & Brown, 2014; Goulson *et al.*, 2018), having undergone a range expansion of approximately 900 km in the 20 years since it was initially recorded (**Figure 1.2**). Therefore, in contrast to bumblebee species experiencing ongoing declines (e.g. Cameron *et al.*, 2011; Dupont *et al.*, 2011), *B. hypnorum* represents a prime example of a highly ecologically successful bumblebee that is currently undergoing a range expansion (in this case, westwards across Europe). Additionally, *B. hypnorum* is of further interest due to its facultatively polyandrous mating system in both continental European and UK populations (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000; Paxton *et al.*, 2001; Brown *et al.*, 2002, 2003; Crowther *et al.*, 2019), which creates within-population, between-colony kin structure variation. The facultatively polyandrous nature of *B. hypnorum* therefore allows the testing of kin-selected predictions relating to when co-operation and conflict should occur during social evolution (Queller & Strassmann, 1998; Ratnieks *et al.*, 2006; Wenseleers & Ratnieks, 2006).

1.4. Thesis aims

Given the above, the UK Tree Bumblebee population represents an illuminating case study in which to investigate: (1) the population genetic factors associated with successful colonisation and subsequent range-expansion; (2) the life-history traits associated with rapid ecological success within a newly-colonised range; (3) the inclusive fitness predictions of reproductive conflict and its resolution within eusocial societies; and (4) the potential contributions and interactions of genetic, life-history and social traits that might help explain *B. hypnorum*'s range-expansion success. Accordingly, the four empirical data chapters in this thesis use a combination of genetic, demographic, and behavioural approaches to investigate these topics, with a particular focus on the population genetics and ecological success of the Tree Bumblebee within the UK, on the influence of life-history traits including relationships with parasites, and on the resolution of reproductive conflict within eusocial societies.

(a) Population genetics of a range-expanding bumblebee

During colonisation events, founding populations are typically subject to a loss of genetic diversity, known as a genetic bottleneck, which may reduce adaptability and hinder the success of the founding population (Nei *et al.*, 1975; Willi *et al.*, 2006). Nonetheless, there are numerous examples of species that have successfully colonised new ranges despite suffering severe bottlenecks on their initial arrival (Estoup *et al.*, 2016; Schrieber & Lachmuth, 2017), a phenomenon known as the genetic paradox of invasion (Allendorf & Lundquist, 2003). Given their haplodiploid method of sex determination, eusocial Hymenoptera should be particularly vulnerable to genetic bottlenecks, given that homozygosity at the sex-determining locus leads to the production of diploid males at the expense of workers (Cook & Crozier, 1995; Hagan & Gloag, 2021). Nonetheless, the eusocial Hymenoptera represent some of the most invasive species worldwide (Chapman & Bourke, 2001; IUCN, 2013), with numerous species succeeding within their new ranges despite suffering severe bottlenecks upon their introduction (Tsutsui *et al.*, 2000; Schmid-Hempel *et al.*, 2007; Arca *et al.*, 2015; Gloag *et al.*, 2016; Schmack *et al.*, 2019; Jones *et al.*, 2020).

Previous research suggested that *B. hypnorum* underwent a severe genetic bottleneck upon its arrival in the UK (Jones & Brown, 2014). Hence, the ecological success of *B. hypnorum* in the UK despite a severe bottleneck has been cited as a prime example of the genetic paradox of invasion (Schrieber & Lachmuth, 2017). However, previous research did not directly measure the genetic diversity of the UK *B. hypnorum* population, nor genetically quantify the frequency of diploid males (Jones & Brown, 2014). Therefore, whether the colonisation of the UK by *B. hypnorum* was associated with a genetic bottleneck and truly represents a genetic paradox of invasion (Schrieber & Lachmuth, 2017) remains to be answered. Hence, using microsatellite genotyping, the aim of **Chapter 2** was to test for the presence of a historic genetic bottleneck and quantify levels of diploid male frequency (an indicator of low genetic diversity) within the UK *B. hypnorum* population, allowing the test of two alternative hypotheses (‘gene flow’ versus ‘bottleneck’ hypotheses) regarding the colonisation of the UK by *B. hypnorum*. This chapter has been published as Brock *et al.* (2021b).

(b) Host-parasite interactions in a range-expanding bumblebee

Parasites are pervasive across ecosystems, and play key roles in regulating the abundance and density of host populations (Anderson, 1978; Albon *et al.*, 2002). Parasites therefore form a key component of the enemy-release hypothesis (Torchin *et al.*, 2003), which predicts that the ecological success of an invasive species within its new range is due to its escape from natural enemies, including parasites, encountered within its native range (Jeffries & Lawton, 1984; Keane & Crawley, 2002). Bumblebees host a broad range of generalist parasites (Bartolomé *et al.*, 2021) that impact both individual- and colony-level fitness traits and may influence population dynamics (e.g. Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2008; Gillespie, 2010). Accordingly, there is special interest in the potential impacts of host-parasite interactions on population trends of both declining and range-expanding bumblebees (Allen *et al.*, 2007; Jones & Brown, 2014; Arbetman *et al.*, 2017).

Previous research has demonstrated similar parasite prevalence and community structure between *B. hypnorum* and five UK native bumblebee species, suggesting that the ecological success of *B. hypnorum* in the UK cannot be attributed to parasite escape (Jones & Brown, 2014). However, while *B. hypnorum* may not have escaped from parasites in its newly colonised range, other parasite-mediated mechanisms may explain its success as a range-expanding bumblebee. Of particular interest is *B. hypnorum*’s potential resistance to the castrating impacts that the parasitic nematode *Sphaerularia bombi* inflicts on multiple bumblebee species (Hasselrot, 1960; Röseler, 2002; Rutrecht & Brown, 2008; Kelly, 2009; Jones & Brown, 2014). Resistance to the castrating impacts of *S. bombi* may represent a factor underlying the success of *B. hypnorum* in the UK but may also have severe impacts on the fitness of *S. bombi* by limiting the ability of the parasite to complete its life cycle. However, quantitative studies detailing interactions between *B. hypnorum* and *S. bombi* are lacking to date. Hence, the aim of **Chapter 3** was to provide the first detailed study of host-parasite interactions between *B. hypnorum* and *S. bombi*, allowing the quantification of queen fitness costs associated with *S. bombi* infection and the reproductive success of *S. bombi* upon infecting the *B. hypnorum* host.

(c) Colony demography and queen lifetime fitness in a range-expanding bumblebee

Despite growing interest in social insects as a model system to understand life-history trade-offs between reproduction and longevity (Korb & Heinze, 2021), relationships between queen longevity and queen reproductive success are little studied in the eusocial Hymenoptera (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015). This is more broadly reflective of the general lack of colony-level, life-history data across the eusocial Hymenoptera, despite the value of such data for understanding social evolution (Tschinkel, 1991; Cole, 2009). In bumblebees, colony demography has been most extensively studied in the common and widespread Buff-tailed Bumblebee, *B. terrestris* (Duchateau & Velthuis, 1988; Beekman & Van Stratum, 1998; Duchateau *et al.*, 2004). However, whether demographic traits observed in *B. terrestris* are applicable to all bumblebee species, and hence represent general insights into the evolution of sociality across all bumblebees, is unknown.

Given its status as a rapidly range-expanding bumblebee, characterisation of colony demography in *B. hypnorum* is of particular interest, since such data may yield insights into the colony-level traits associated with ecological success. Hence, the aim of **Chapter 4** was to present the first comprehensive characterisation of colony demography in *B. hypnorum* and compare colony-level traits to those of the well-studied *B. terrestris* to elucidate potential factors underlying *B. hypnorum*'s success in the UK. Further, the collection of such demographic data also allowed tests for associations between queen lifetime reproductive success and multiple individual- and colony-level traits, such as queen longevity, colony size, and colony longevity, providing further insight into the impact of sociality on lifetime fitness.

(d) Reproductive conflict resolution in a eusocial society

The high levels of co-operation observed within eusocial societies have led to them being described as ‘superorganisms’ (Hölldobler & Wilson, 2009). However, given that eusocial societies are usually familial rather than clonal in their kin structure, fitness optima between individuals do not always align, leading to reproductive conflict (as predicted by inclusive fitness theory) arising between individuals (Hamilton, 1964b; Trivers & Hare, 1976; Queller & Strassmann, 1998). Conflict over male parentage is widespread across the eusocial Hymenoptera since, under haplodiploid sex determination, workers are able to lay haploid, male eggs to which both egg-layers and their full sisters are more highly related than to queen-produced males (Bourke, 1988; Hammond & Keller, 2004). Male production conflict represents a dissolutive force during social evolution (Bourke, 2011a) but can be resolved through policing, a coercive mechanism whereby queens and/or workers limit worker reproduction through aggression towards reproductive workers and selective destruction of their eggs (Ratnieks, 1988; Ratnieks *et al.*, 2006). Many factors may select for worker policing, including queen polyandry (Crozier & Pamilo, 1996), increased colony productivity (Hartmann *et al.*, 2003; Teseo *et al.*, 2013), or future worker reproduction (Wenseleers *et al.*, 2005; Zanette *et al.*, 2012), and act synergistically to reduce male production conflict. However, to date, interactions between polyandry, reproductive conflict, policing rates, and colony productivity have yet to be thoroughly examined.

In this respect, due to its facultatively polyandrous mating system (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000; Paxton *et al.*, 2001; Brown *et al.*, 2002, 2003; Crowther *et al.*, 2019), *B. hypnorum* offers an excellent study system to study relationships between relatedness, conflict resolution, and colony productivity. Previous studies found differing levels of worker-produced males in *B. hypnorum* colonies (Paxton *et al.*, 2001; Brown *et al.*, 2003), leading to contrasting conclusions about the existence and effectiveness of policing mechanisms in *B. hypnorum* colonies. However, these early studies focused on quantifying male parentage and did not quantify rates of egg-laying or policing by the queen or her workers, which represent two important mechanisms underlying conflict resolution in eusocial hymenopteran societies (Ratnieks *et al.*, 2006). Hence, the primary aim of **Chapter 5** was to present the first quantitative behavioural and genetic analyses of male production conflict and its outcomes (in terms of male parentage) in *B. hypnorum*. Secondly, I aimed to relate levels of conflict to colony kin structure and colony productivity, allowing the test of hypotheses that worker policing occurs as a function of queen mating frequency and/or as a method of increasing colony productivity.

Taken as a whole, this thesis makes novel contributions to our understanding of the genetic and ecological features underlying ecological success in a range-expanding bumblebee, as well as providing insight into the mechanisms regulating kin-selected conflict in eusocial societies. A synthesis of the findings presented in the thesis is provided in **Chapter 6**.

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Chapter 2

No severe genetic bottleneck in a rapidly range-expanding bumblebee pollinator

A newly eclosed Tree Bumblebee (*Bombus hypnorum*) male.

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CHAPTER 2

No severe genetic bottleneck in a rapidly range-expanding bumblebee pollinator

2.1. Abstract

Genetic bottlenecks can limit the success of populations colonising new ranges. However, successful colonisations can occur despite bottlenecks, a phenomenon known as the genetic paradox of invasion. Eusocial Hymenoptera such as bumblebees (*Bombus* spp.) should be particularly vulnerable to genetic bottlenecks, since homozygosity at the sex-determining locus leads to costly diploid male production. The Tree Bumblebee (*B. hypnorum*) has rapidly colonised the UK since 2001 and has been highlighted as exemplifying the genetic paradox of invasion. Using microsatellite genotyping, combined with the first genetic estimates of diploid male production in UK *B. hypnorum*, we tested two alternative genetic hypotheses ('bottleneck' and 'gene flow' hypotheses) for *B. hypnorum*'s colonisation of the UK. We found that the UK population has not undergone a recent severe genetic bottleneck and exhibits levels of genetic diversity falling between those of widespread and range-restricted *Bombus* species. Diploid males occurred in 15.4% of reared colonies, leading to an estimate of 21.5 alleles at the sex-determining locus. Overall, the findings show that this population is not bottlenecked, instead suggesting that it is experiencing continued gene flow from the continental European source population with only moderate loss of genetic diversity, and does not exemplify the genetic paradox of invasion.

2.2. Introduction

Colonisation and invasion events involve changes in population size, with founding populations typically representing a subset of the source population. This reduction in population size creates a sampling effect on alleles, reducing genetic diversity (Nei *et al.*, 1975). Such a phenomenon, known as a genetic bottleneck, can lead to reduced adaptive potential (Willi *et al.*, 2006), inbreeding and subsequent loss of heterozygosity (Charlesworth & Charlesworth, 1987), and stochastic increases in the frequency of deleterious alleles (Lynch *et al.*, 1995). In turn, these processes may reduce the fitness of a founding population and thereby hinder its establishment and spread across new ranges. However, there are numerous examples of species that have successfully colonised new ranges after undergoing severe genetic bottlenecks during their initial introduction (Estoup *et al.*, 2016; Schrieber & Lachmuth, 2017), a phenomenon known as the genetic paradox of invasion (Allendorf & Lundquist, 2003). Hence, despite counter-cases (Roman & Darling, 2007; Estoup *et al.*, 2016), the relationship between colonisation success and levels of genetic diversity in founding populations remains to be fully resolved.

Genetic bottlenecks are potentially even more harmful in the Hymenoptera (ants, bees, wasps, and sawflies) due to single-locus complementary sex determination (*sl*-CSD; Hagan and Gloag, 2021). In *sl*-CSD, allelic diversity at a single locus, combined with haplodiploidy, determines an individual's sex (Cook, 1993). Specifically, diploid individuals heterozygous at the sex-determining locus develop as females whereas haploid individuals hemizygous at the sex-determining locus develop as males (**Figure 2.1**). Under low genetic diversity and/or inbreeding, there will be an increasing frequency of mating pairs sharing an allele at the sex-determining locus ('matched mating'). In such cases, 50% of diploid offspring produced will be homozygous at the sex-determining locus and therefore develop as diploid males (Cook & Crozier, 1995; **Figure 2.1**).

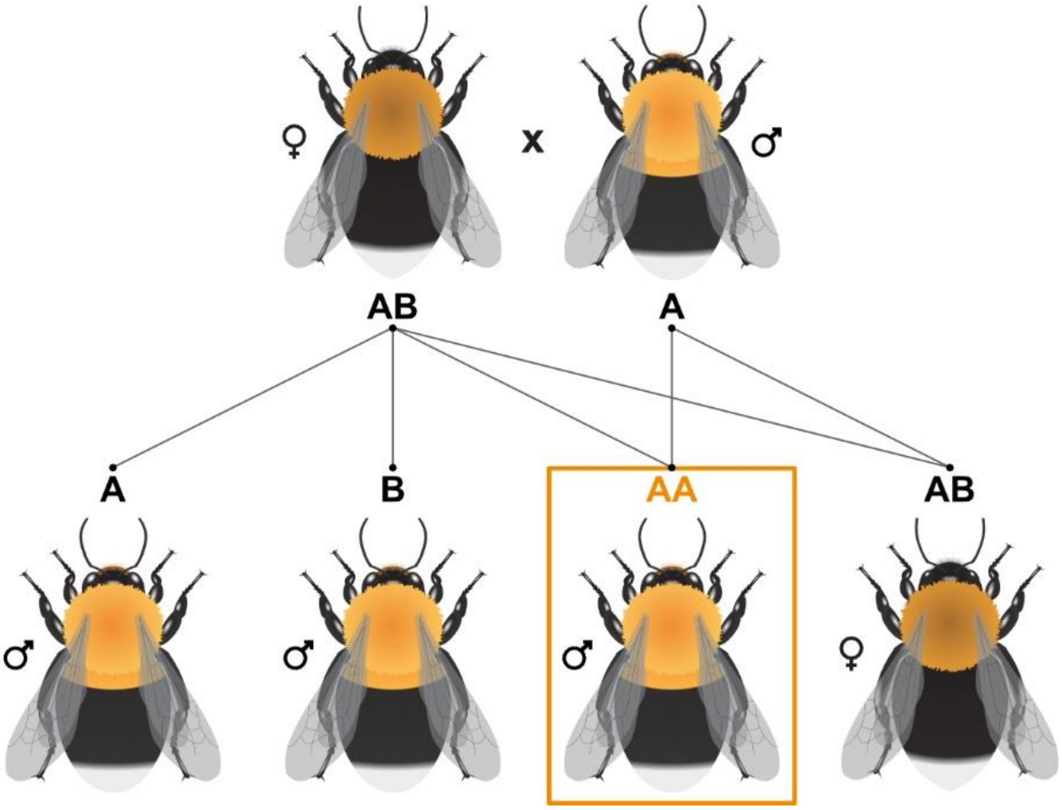

Figure 2.1. Single-locus complementary sex determination (sl-CSD) and diploid male production in the Hymenoptera. In sl-CSD, allelic diversity at the sex-determining locus, combined with haplodiploidy, determines sex. Individuals heterozygous at the sex-determining locus (genotype **AB**) develop as females, whereas hemizygous individuals (genotype **A** or **B**) develop as males. Low genetic diversity and/or inbreeding leads to an increased frequency of matings occurring between partners that share a sex-determining allele (allele **A** in the above example). Here, half of the diploid offspring produced are homozygous at the sex-determining locus (genotype AA), subsequently developing as diploid males (orange box).

Diploid male production (DMP) is costly since diploid males are inviable or sterile (Beye *et al.*, 2003; Armitage *et al.*, 2010) or produce inviable or sterile triploid offspring (Duchateau & Mariën, 1995; Liebert *et al.*, 2005). These negative fitness impacts are exacerbated in colonies of eusocial Hymenoptera (all ants, bees, and wasps with a worker caste), in which diploid males replace half of the potential workers but do not contribute to colony productivity (Cook & Crozier, 1995; Hagan & Gloag, 2021). Accordingly, DMP reduces colony founding success and productivity in both ants and bumblebees (Ross & Fletcher, 1986; Whitehorn *et al.*, 2009; Gosterit, 2016; Bortolotti *et al.*, 2020). In addition, a high frequency of DMP represents a strong indicator of a genetic bottleneck (Buttermore *et al.*, 1998; Darrouzet *et al.*, 2015). Despite this, eusocial Hymenoptera account for some of the most invasive species worldwide (Chapman & Bourke, 2001; IUCN, 2013), with several cases occurring in which invasive populations have undergone severe bottlenecks (Tsutsui *et al.*, 2000; Schmid-Hempel *et al.*, 2007; Arca *et al.*, 2015; Gloag *et al.*, 2016; Schmack *et al.*, 2019). For example, the Yellow-legged Hornet (*Vespa velutina*) has successfully colonised much of southern Europe following a founding event inferred to involve a single multiply-mated queen (Arca *et al.*, 2015).

Eusocial Hymenoptera perform essential services across natural and farmed ecosystems (Del Toro *et al.*, 2012; Elizalde *et al.*, 2020; Brock *et al.*, 2021a). In particular, eusocial bees, including bumblebees and the honeybee *Apis mellifera*, represent some of the most important insect pollinators of food crops and wild plants (Klein *et al.*, 2007), a role that is threatened by their widespread declines (e.g. Klein *et al.*, 2007; Williams *et al.*, 2009; Cameron *et al.*, 2011). However, at regional scales, some bee species are expanding their ranges, through either human transportation or natural colonisation events (Goulson & Williams, 2001; Hingston, 2006; Madjidian *et al.*, 2008; Gloag *et al.*, 2016; Biella *et al.*, 2020; Rollin *et al.*, 2020). Given concerns over maintaining bee populations, there is special interest in determining the relationship between ecological success and the genetic consequences of bottlenecks, mediated by *sl*-CSD, in range-expanding eusocial bee species (Schmid-Hempel *et al.*, 2007; Gloag *et al.*, 2016).

The Tree Bumblebee (*Bombus hypnorum*) represents a highly successful range-expanding bumblebee. Having been initially recorded in southern England in 2001 (Goulson & Williams, 2001), apparently as a natural colonist, it has rapidly increased in range and abundance to become one of the most widespread and common UK bumblebee species (Crowther *et al.*, 2014, 2019; Jones and Brown, 2014; **Figure 1.2**). Historically, the range of *B. hypnorum* extends across continental Europe and Asia (Goulson & Williams, 2001; Rasmont *et al.*, 2015). Hence, it seems likely that *B. hypnorum* arrived in the UK from the closest neighbouring area of its pre-2001 range, northern France (Goulson & Williams, 2001; Rasmont *et al.*, 2015).

A previous study suggested that *B. hypnorum* underwent a severe genetic bottleneck on its arrival in the UK, with male production in the first brood (indicative of DMP, as first broods are usually composed of workers alone) being observed in three of 13 colonies reared from field-collected queens (Jones & Brown, 2014). These data were used to estimate that the sex-determining locus in the UK *B. hypnorum* population has four alleles and that the founding population consisted of one or two multiply-mated queens (Jones & Brown, 2014). Consequently, the successful establishment and spread of *B. hypnorum* in the UK despite an apparently severe genetic bottleneck has been cited as a prime example of the genetic paradox of invasion (Schrieber & Lachmuth, 2017).

However, the previous work (Jones & Brown, 2014) did not confirm DMP genetically, nor account for facultative polyandry (multiple mating by queens) in *B. hypnorum* (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000; Paxton *et al.*, 2001; Brown *et al.*, 2003; Crowther *et al.*, 2019), potentially leading to an inaccurate estimate of allelic diversity at the sex-determining locus (Adams *et al.*, 1977). Recently, up to 11 alleles were found at neutral microsatellite loci in workers of a UK *B. hypnorum* population, in which queen mating frequency was estimated at 1.7 mates per queen (Crowther *et al.*, 2019), suggesting a founding population of greater than two queens. Moreover, recording data show that *B. hypnorum* has expanded its range westwards within continental Europe across Germany and Belgium from the middle of the 20th century (Wagner, 1937; Rasmont, 1989; Rollin *et al.*, 2020), suggesting that colonisation of the UK may represent part of an ongoing, large-scale range expansion in Europe. Hence, whether colonisation of the UK by *B. hypnorum* truly exemplifies the genetic paradox of invasion is uncertain. This suggestion is also consistent with the findings of a recent

RAD-seq study that showed similar levels of genetic diversity and no evidence of structuring between six UK *B. hypnorum* populations and one in northern France (Huml *et al.*, 2021).

Therefore, we defined two contrasting genetic hypotheses to characterise the mode of colonisation of the UK by *B. hypnorum*. Under the 'bottleneck hypothesis', a small number of individuals founded the entire UK population in a single, chance event (Jones & Brown, 2014). This hypothesis predicts that the UK *B. hypnorum* population will show low genetic diversity, evidence of a recent severe genetic bottleneck (i.e. at the time of colonisation), and high levels of DMP. In contrast, under the 'gene flow hypothesis', colonisation of the UK by *B. hypnorum* represented part of an ongoing, large-scale westward range expansion, with a larger founding population and subsequent continued immigration from continental European populations (Crowther, 2018). This hypothesis predicts that the UK *B. hypnorum* population will show high genetic diversity, no recent severe genetic bottleneck, and low levels of DMP.

Using a panel of previously characterised polymorphic microsatellite loci (Crowther *et al.*, 2019), we sought to discriminate between these two hypotheses and so establish whether *B. hypnorum*'s colonisation of the UK truly represents a genetic paradox of invasion. To this end, within a representative UK population of *B. hypnorum*, we pursued two aims. First, we quantified genetic diversity in workers (using data from Crowther *et al.*, 2019) and males to perform the first test of whether the UK *B. hypnorum* population has undergone a severe genetic bottleneck relative to established UK bumblebee species. Second, because determining levels of DMP provides a powerful independent means of estimating levels of genetic diversity in eusocial Hymenoptera, we estimated the frequency of diploid males and allelic diversity at the sex-determining locus genetically for the first time in the UK *B. hypnorum* population.

2.3. Methods

(a) Genetic diversity and bottleneck analyses

Worker sample collection and genotyping

Workers from a population of *B. hypnorum* in Norwich, Norfolk, UK, were used to test for a recent reduction in effective population size (bottleneck), i.e. one occurring at the time of colonisation (Goulson & Williams, 2001). A total of 675 *B. hypnorum* workers were sampled non-lethally (Holehouse *et al.*, 2003) from a 2 km^2 area across two consecutive summers (2014: n = 398; 2015: n = 277). Of these, 645 workers (2014: n = 375; 2015: n = 270) were genotyped at up to 14 microsatellite loci (median = 11 loci), following methods described in detail in Crowther *et al.* (2019) and in the supplementary materials for Brock *et al.* (2021b).

Genetic diversity and bottleneck evaluation

All 645 worker genotypes from Crowther *et al.* (2019) were used to estimate measures of genetic diversity, i.e. the number of alleles per locus, mean allelic richness (A_R), and mean observed (H_o) and expected heterozygosity (H_e).

Using single workers randomly selected from each of 89 independent clusters among the worker genotypic data identified with COLONY v2 (Jones & Wang, 2010) (see online supplementary materials for Brock *et al.*, 2021b), two different methods were employed to test for a bottleneck in the *B. hypnorum* study population. The first was a sign test implemented in the program BOTTLENECK 1.2.02 (Piry *et al.*, 1996), in which an excess of expected heterozygosity over that expected under mutation-drift equilibrium suggests that a recent reduction in population size has occurred (Cornuet & Luikart, 1996). The second test calculated the M-ratio (Garza & Williamson, 2001) across loci for the *B. hypnorum* study population. The M-ratio defines the ratio between allelic diversity and allele size range at a locus, with bottlenecks leading to the stochastic loss of rare alleles and a subsequent reduction of the M-ratio (Garza & Williamson, 2001). Under mutation-drift equilibrium, an M-ratio of less than 0.7 can be interpreted as evidence of a historical population reduction, a signal that may persist for more than 100 generations (Garza & Williamson, 2001).

To provide a set of comparative M-ratios in established UK *Bombus* species, M-ratios were also calculated for UK populations of the five species, *B. hortorum*, *B. lapidarius*, *B. pascuorum*, *B. ruderatus*, and *B. terrestris* (Dreier *et al.*, 2014). These 'reference' *Bombus* species have not undergone range expansions within the UK, and, except for the scarce *B. ruderatus*, are common and widespread. Therefore, M-ratios calculated for these species provided null values against which the M-ratio calculated for *B. hypnorum* was compared. Hence, if *B. hypnorum* experienced a severe genetic bottleneck upon its colonisation of the UK, it would be expected to exhibit a lower M-ratio than the reference *Bombus* species. Full details of both tests can be found in the supplementary materials for Brock *et al.* (2021b).

(b) Diploid male production and allelic diversity at the sex-determining locus

Male sample collection and genotyping

To estimate levels of DMP in *B. hypnorum*, males were sampled from two sources. First, 380 male pupae were sampled from 20 mature *B. hypnorum* colonies collected in the field in Norfolk and Suffolk, UK, over two consecutive years (2017: $n_{colonies} = 17$, $n_{male\ pupae} = 337$; 2018: $n_{colonies} = 3$, $n_{male\ pupae} = 43$). These 20 colonies each provided 7–24 randomly sampled male pupae for genotyping (see **Table S1** in Brock *et al.*, 2021b).

Second, to allow for survivorship biases in field-collected nests (as detailed in the supplementary materials for Brock *et al.*, 2021b), adult males were sampled from *B. hypnorum* colonies reared from 107 queens collected from field sites in Surrey, Greater London, and Norfolk (Norwich), UK, during spring 2018 (see **Table S2** in Brock *et al.*, 2021b). In total, 37 of the 107 field-collected queens reared at least one adult offspring (see **Table S3** in Brock *et al.*, 2021b), with nine colonies producing only workers, six colonies producing only males, and 22 colonies producing both workers and males. Of the 28 colonies that produced males, 12 were assigned as 'first-brood male' producers, which were defined as colonies in which either any males eclosed within one week of first worker eclosion ($n_{colonies}$ = 6) or only males and no workers eclosed ($n_{colonies}$ = 6) (mean [range] *n* of first-brood males produced per colony = 2 [1-7]); the remaining 16 were assigned as 'late male' producers, which were defined as colonies in which all males eclosed later than one week after first worker eclosion (see **Figure S3** and **Table S2** in Brock *et al.*, 2021b). From the 28 male-producing colonies, a total of 232 adult males were sampled for genotyping (see **Table S3-S5** in Brock *et al.*, 2021b), comprising 25 first-brood males, which were defined as males that either eclosed within one week of first worker eclosion or were produced by colonies producing no workers, and 207 late males, which were defined as males that eclosed later than one week after first worker eclosion.

All sampled males (*n* = 612, i.e. 380 pupal males from field-collected nests plus 232 adult males from colonies reared from field-collected queens) were genotyped at the same microsatellite loci as were used for the 2014/15 worker samples described above. However, because in males one locus (BTMS0132) proved monomorphic, data analysis in males was based on a median (range) of 13 (3-13) polymorphic loci.

Estimation of levels of diploid male production

Diploid males were assigned as those phenotypic males that were heterozygous at two or more microsatellite loci across two independent rounds of genotyping (as detailed in the supplementary materials for Brock *et al.*, 2021b). All males accepted as diploid (50 of 612 males genotyped) were heterozygous at a mean (range) of 5.5 (2-9) loci.

The diploid male data were then used to produce three estimates of the frequency of colonies exhibiting DMP: (1) from the field-collected colonies; (2) from the colonies reared from field-collected queens; and (3) from the latter colonies after correcting for sampling error arising when males were sampled for genotyping (as detailed in the supplementary materials for Brock *et al.*, 2021b).

Estimation of allelic diversity at the sex-determining locus

By applying the formula of Adams *et al.* (1977) and an approach accounting for facultative polyandry, our data on the frequency of colonies exhibiting DMP and previous data on the frequency distribution of queen mating frequencies in the main study *B. hypnorum* population (Crowther *et al.*, 2019) were used to estimate the number of alleles at the sex-determining locus (as detailed in the supplementary materials for Brock *et al.*, 2021b). Boundary values were calculated by assuming either (1) 100% single mating of queens or (2) 50% double mating and 50% triple mating of queens, as increasing levels of polyandry have the greatest effect on the estimated number of alleles at this locus (see the supplementary materials for Brock *et al.*, 2021b).

All data analyses were carried out using R v4.0.1 (R Core Team, 2020), unless stated otherwise.

2.4. Results

(a) Genetic diversity and bottleneck analyses

Across the 645 workers and 14 microsatellite loci, the median number (range) of alleles per locus was 5.0 (3–11), mean allelic richness was 5.9, and mean observed and expected heterozygosity were, respectively, 0.48 and 0.51.

The sign test found no evidence of a recent bottleneck, as the number of loci that had an excess of heterozygosity (7.0 of 14 loci) was not significantly different from that expected (7.5 of 14 loci; $p = 0.49$).

The M-ratio (mean ± standard error) was 0.38 ± 0.05. This fell within the range of the mean M-ratios for the five reference *Bombus* species (**Figure 2.2**), and overall, across these species plus *B. hypnorum*, there was no significant difference in mean M-ratio (ANOVA, $F_{5, 31.9} = 1.95$, $p = 0.11$). Unexpectedly, all *Bombus* species exhibited M-ratios under 0.7 (**Figure 2.2**), indicating some support for historical population reductions in all these populations. However, the overall conclusion from both the sign test and the M-ratio analysis was that there was no strong evidence for a recent severe genetic bottleneck in the UK *B. hypnorum* population relative to UK populations of other *Bombus* species.

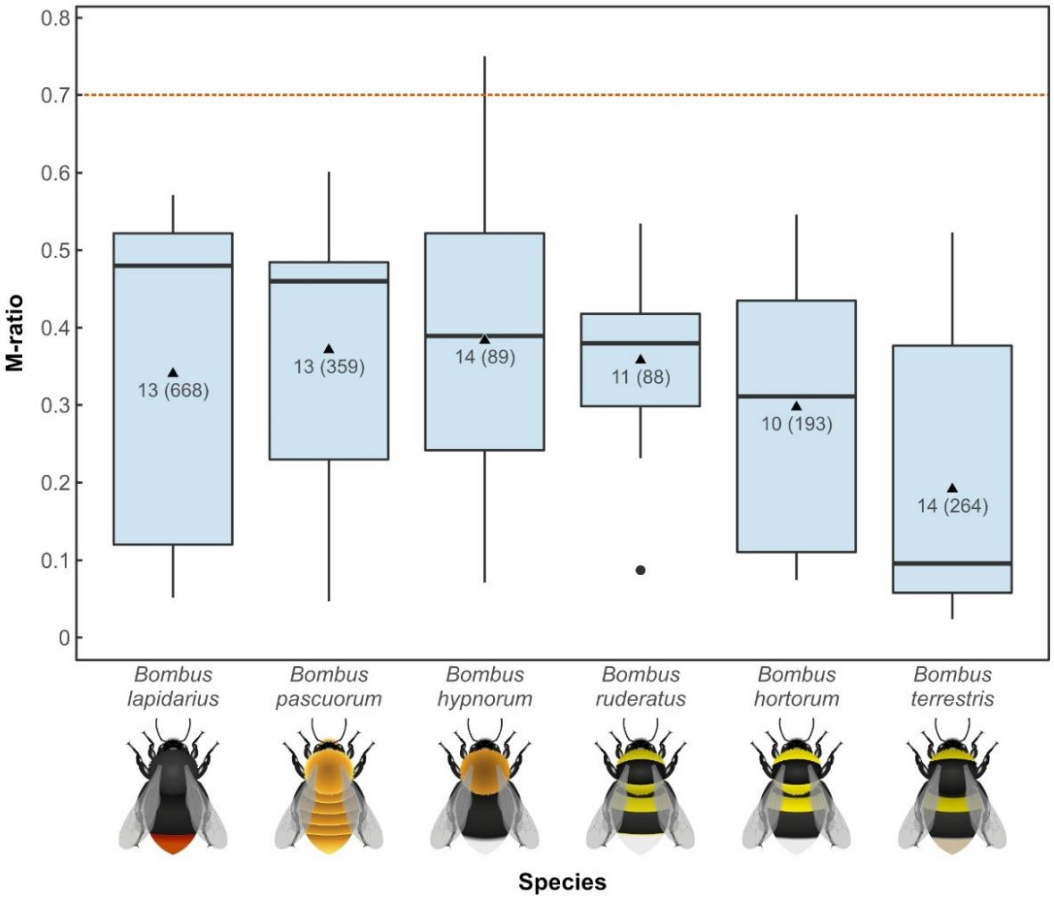

Figure 2.2. M-ratios across microsatellite loci for the main UK study population of *Bombus hypnorum* (current study) and for single UK populations of five other UK *Bombus* species (calculated using data from Dreier et al., 2014) in order (left to right) of decreasing medians. Thick horizontal bars, median; orange triangles, mean; boxes, interquartile range (IQR); whiskers, range (not including outliers); filled circles, outliers, defined as points more than 1.5 IQR below lower quartile. Numbers within the boxes, number of microsatellite loci and, in parentheses, number of unrelated workers used to calculate the M ratios for each species. Orange dashed line at 0.7, M-ratio threshold below which a historical population reduction is hypothesised to have occurred.

(b) Diploid male production and allelic diversity at the sex-determining locus

Levels of diploid male production

Across the 612 males and 13 microsatellite loci, the median (range) number of alleles per locus was 5.0 (3-8), and mean allelic richness was 5.3 (see **Table S6** in Brock *et al.*, 2021b), broadly matching the results from the worker genotypes.

All 20 field-collected colonies produced diploid offspring (workers and/or gynes; see **Table S1** in Brock *et al.*, 2021b) and diploid male pupae were found in one of them (5%), accounting for 4.5% of all genotyped male pupae (**Figure 2.3A**).

Genotyping of adult males from the 37 colonies reared from field-collected queens showed that, of the 32 colonies that produced diploid offspring (including one male-only producing colony that produced a diploid male), five (15.6%) produced diploid males (see **Table S3-S5** in Brock *et al.*, 2021b). These five DMP colonies comprised four of 12 (33.3%) first-brood male producing colonies and one of 16 (6.3%) late-male producing colonies (**Figure 2.3B-C**). At the level of individual males, 4 of 25 (16.0%) first-brood males and 29 of 207 (14.0%) late males were found to be diploid (see **Table S3** in Brock *et al.*, 2021b), with diploid males accounting for 14.2% of all genotyped males. Of the 26 colonies reared from field-collected queens, producing diploids, and retained after correcting for sampling error, 4 (15.4%) produced diploid males (see **Table S3-S5** in Brock *et al.*, 2021b).

Overall, therefore, 15.4-15.6% of colonies reared from field-collected queens produced diploid males. Moreover, given that male diploidy was genetically confirmed in only 33.3% of first-brood male producing colonies and 16.0% of first-brood males, first-brood male production was a poor indicator of DMP, as previously it has been assumed that 100% of first-brood male producing colonies would be diploid male producing colonies and 100% of first-brood males would be diploid males.

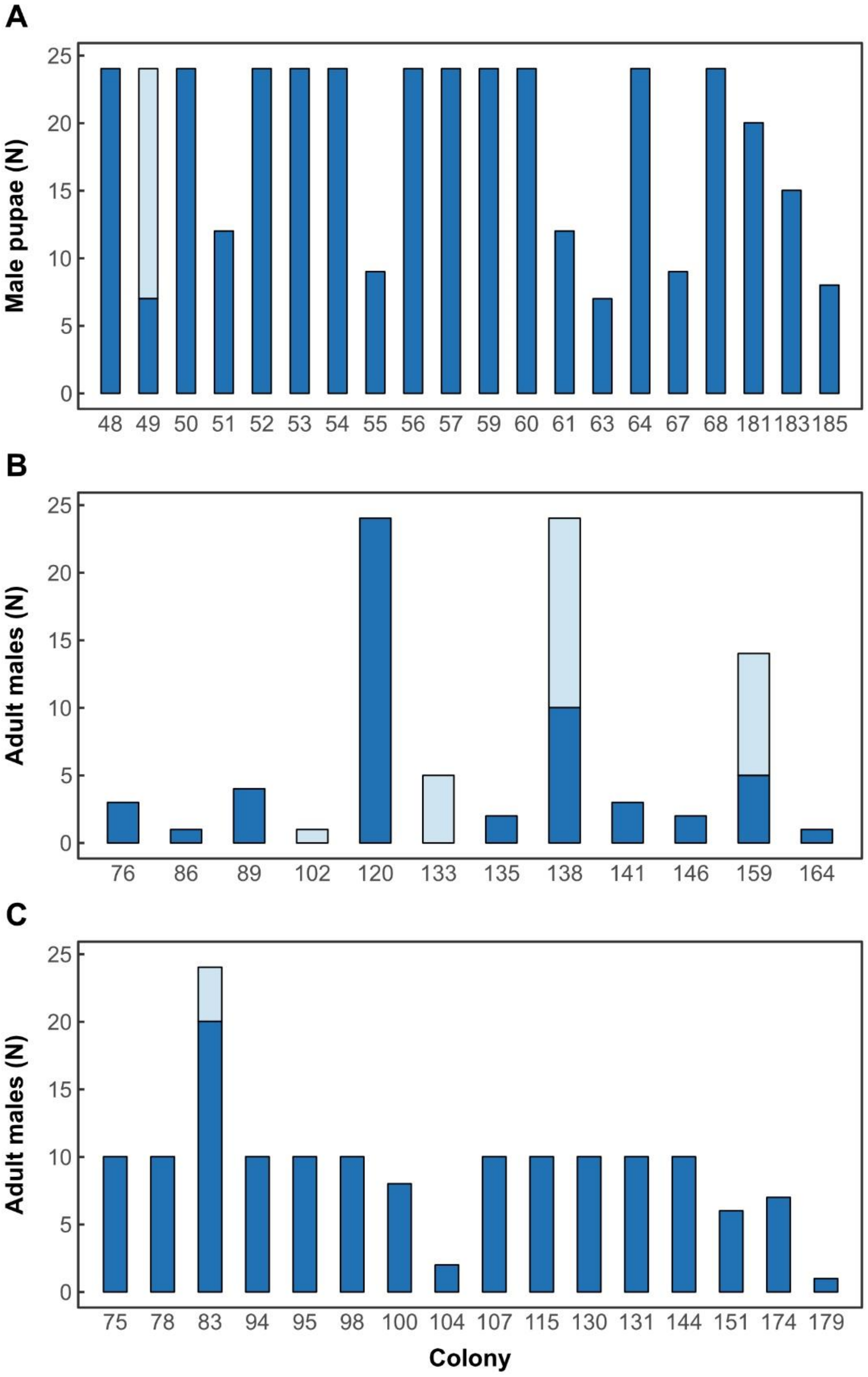

Figure 2.3. Numbers of haploid (dark blue bars) and diploid (light blue bars) pupal or adult males verified by genotyping at up to 13 microsatellite loci in: **(A)** 20 mature, field-collected *Bombus hypnorum* colonies; **(B)** 12 first-brood male-producing *B. hypnorum* colonies reared from field-collected queens; and **(C)** 16 late male-producing *B. hypnorum* colonies reared from field-collected queens. Numbers on *x* axes are individual colony identification numbers.

Allelic diversity at the sex-determining locus

Estimates of the proportion of colonies exhibiting DMP for field-collected colonies, colonies reared from field-collected queens, and colonies reared from field-collected queens after correcting for sampling error were 1/20, 5/32, and 4/26, respectively, or 0.05, 0.156, and 0.154, respectively (as above). Combined with published data on the relative frequencies of singly, doubly, and triply mated queens in the main study population (Crowther *et al.*, 2019), these yielded matched mating frequency estimates of 0.029, 0.094, and 0.093, respectively, and hence estimates of the number of alleles at the sex-determining locus of 69.0, 21.3, and 21.5, respectively (as detailed in the supplementary materials for Brock *et al.*, 2021b). Taking the last estimate to be the most accurate, and adding the calculated boundary values, led to an estimated number (with boundary values) of 21.5 (13.0-30.8) alleles at the sex-determining locus in the UK *B. hypnorum* population.

2.5. Discussion

We analysed genotypic data from a UK population of the Tree Bumblebee (*B. hypnorum*) to discriminate between two hypotheses for colonisation of the UK by this range-expanding species. The bottleneck hypothesis (small founding population in a single, chance colonisation event) predicted low genetic diversity, a recent severe genetic bottleneck, and high levels of diploid male production (DMP). The gene flow hypothesis (larger founding population and subsequent continued immigration from continental European populations) predicted high genetic diversity, no recent severe genetic bottleneck, and low levels of DMP. Our results showed relatively high allele numbers at microsatellite loci and (as estimated from DMP levels) at the sex-determining locus, no evidence of a recent severe genetic bottleneck, and relatively low levels of DMP. Therefore, they do not support the bottleneck hypothesis and instead support the gene flow hypothesis.

Consequently, although *B. hypnorum* has rapidly and successfully expanded its range, it does not represent an example of the genetic paradox of invasion.

(a) Genetic diversity and bottleneck analyses

Previous evidence suggested that the founding UK *B. hypnorum* population numbered as few as one or two multiply-mated queens (Jones & Brown, 2014). However, two queens mated with a mean 1.7 males each (Crowther *et al.*, 2019) would yield a maximum of 7.4 alleles at any locus. This number of alleles is lower than the maximum numbers of alleles found at microsatellite loci in the combined data from workers in Crowther *et al.* (2019) and males in the current study (e.g. 11, 9, 9, and 8 alleles at the loci BTMS0125, B10, BL03, and BTERN02, respectively). Therefore, the allele number data do not support the UK *B. hypnorum* population having been founded in a single event by as few as two multiply-mated queens.

Expected heterozygosity and allelic richness at microsatellite loci in the study *B. hypnorum* population (H_e = 0.51, A_R = 5.9) were both higher than values reported in Belgian and Estonian *B. hypnorum* populations for which comparable data exist (Belgium: H_e = 0.37-0.39, A_R = 1.94-2.03; Estonia: H_e = 0.33, A_R = 1.94; Maebe *et al.*, 2019), while Huml *et al.* (2021) found similar sequence-level diversity between UK *B. hypnorum* populations and a French population. In addition, expected heterozygosity in the study *B. hypnorum* population was, on average, intermediate between values found by previous studies in common, established, and widespread European *Bombus* species and scarce, range-restricted, and/or declining species (see **Table S8** in Brock *et al.*, 2021b). Specifically, it was lower than values for 7/8 populations of common species, and higher than those for 7/8 populations of scarce declining species (see **Table S8** in Brock *et al.*, 2021b). Combined, these genetic diversity comparisons again support the lack of a severe bottleneck in the colonisation of the UK by *B. hypnorum*.

Given that *B. hypnorum* is rapidly expanding its range across the UK, and possibly recently did so across north-western Europe (Wagner, 1937;

Rasmont, 1989; Rollin *et al.*, 2020), one might expect some loss of genetic diversity to occur, since the leading edge of a dispersal front is subject to a loss of alleles and heterozygosity as it moves further from the source population (Ibrahim *et al.*, 1996). This is especially true under leptokurtic dispersal (Ibrahim *et al.*, 1996), where small numbers of long-distance dispersers found new populations, as appears likely to be the case for *B. hypnorum* queens in the UK (Crowther, 2018). The fact that the UK *B. hypnorum* study population shows a level of expected heterozygosity lower than values found in common European *Bombus* species provisionally supports this idea. However, the idea requires full testing by sampling a series of *B. hypnorum* populations across the UK and continental Europe.

Bombus species are annual insects that typically undergo one generation per year. Some, including *B. hypnorum*, have been suggested to exhibit facultative bivoltinism, i.e. two colony cycles (and hence two generations) per year (Edwards & Jenner, 2005), but there is little evidence that the second generation produces many new queens. Therefore, an estimated minimum of 14-15 generations passed between *B. hypnorum*'s colonisation of the UK in or shortly before 2001 and the worker sampling in the current study. Based on previous power analyses, our sign test was sufficiently powerful to have detected a bottleneck 0.25N to 2.5N generations after the initial bottleneck, where N equals the founding population size (number of diploid individuals) immediately after a putative bottleneck (Cornuet & Luikart, 1996). Therefore, the sign test should have detected a bottleneck of 6-60 diploid individuals (15 generations/2.5 = 6, and 15 generations/0.25 = 60). As the sign test found no evidence of a bottleneck, it is therefore unlikely that the founding population size was smaller than 60 diploid individuals (120 haploid genomes). In haplodiploid eusocial Hymenoptera, this is equivalent to either 40 singly-mated or 30 doubly-mated queens. These calculations again reject the bottleneck hypothesis and instead support the gene flow hypothesis, since, if colonisation of the UK was part of a large-scale westward range expansion in *B. hypnorum*, one would expect the number of immigrating queens in the founding year and in each subsequent year to have been relatively high.

The M-ratio analysis found no evidence for a level of historical population reduction in *B. hypnorum* greater than the levels found in five other UK *Bombus* species (**Figure 2.2**). Unexpectedly, M-ratios for all tested *Bombus* species, including *B. hypnorum*, fell below the mutation-drift equilibrium threshold of 0.7 (**Figure 2.2**), suggesting that the sampled populations of all species have undergone some degree of historical population reduction, potentially up to approximately 100 generations ago (Garza & Williamson, 2001). Reasons for this are unknown, as data on *Bombus* distribution and abundances within the UK are mostly limited to recent decades (Goulson *et al.*, 2008). Conceivably, the finding points to former population reductions in all *Bombus* species at lowland agricultural sites (Dreier *et al.*, 2014), perhaps associated with historical changes in agricultural practices. Regardless, the conclusion remains that, in the recent UK *B. hypnorum* population, there was no evidence of a bottleneck more intense than in populations of long-established UK *Bombus* species.

(b) Diploid male production and allelic diversity at the sex-determining locus

In the study population of *B. hypnorum*, frequencies of DMP colonies were 5% for mature, field-collected colonies and 15.4-15.6% for colonies reared from field-collected queens. Given observed levels of facultative polyandry in the main study population (Crowther *et al.*, 2019), these values equated to matched mating frequencies of 2.9% and 9.3-9.4%, respectively. Genetic studies of *B. hypnorum* from continental Europe found no diploid males. However, in these studies, colony sample sizes were lower, i.e. 13 colonies (Paxton *et al.*, 2001) or 10 colonies (Brown *et al.*, 2003), such that comparisons with the UK data return no statistically significant difference in DMP frequencies between UK and continental Europe populations (totals of 6/52 DMP colonies versus 0/23 DMP colonies, respectively: Fisher's exact test: $p = 0.169$). Therefore, at most, DMP frequency is only moderately higher in the UK than in continental European *B. hypnorum* populations. This is again consistent with the gene flow hypothesis, qualified by some loss of genetic diversity having occurred at the dispersal front represented by the UK *B. hypnorum* population.

Previously, a frequency of DMP colonies of 23.1% (3 of 13 colonies) was reported in a UK *B. hypnorum* population (Jones & Brown, 2014), but this was based on the assumption that all first-brood male producing colonies exhibited DMP. However, our findings demonstrate that only 33.3% of such colonies exhibit DMP (**Figure 2.3B**). If this was the case in the first-brood male producing colonies in Jones and Brown (2014), and 6.3% of the other colonies in that study exhibited DMP (as in the current study), then the frequency of DMP colonies in the previous study can be estimated as 12.5% (from $[(0.333 \times 3) + (0.063 \times 10)] / 13 = 0.125$), consistent with the values estimated in the current study.

High DMP frequencies are characteristic of populations of eusocial Hymenoptera known from other evidence to have suffered severe bottlenecks during the colonisation of new ranges. For example, the French *Vespa velutina* population was founded by a single polyandrous queen (Arca *et al.*, 2015), and DMP is observed in 48.3% of field-collected nests (Darrouzet *et al.*, 2015). Similarly, the Tasmanian *B. terrestris* population was founded by two monandrous queens (Schmid-Hempel *et al.*, 2007), and DMP was inferred in 50% of colonies reared from field-collected queens (Buttermore *et al.*, 1998). Correspondingly, the relatively lower levels of DMP observed in the UK *B. hypnorum* population do not support the occurrence of a severe genetic bottleneck.

The estimated number of alleles at the sex-determining locus in the UK *B. hypnorum* population (with boundary values calculated by assuming 100% single mating or 50% double mating and 50% triple mating of queens) was 21.5 (13.0 - 30.8) alleles. This estimate is consistent with the conclusion from the microsatellite data that the UK *B. hypnorum* population has not undergone a severe bottleneck, with even the lower bound exceeding the previous estimate of this number (Jones & Brown, 2014) by over three-fold. Based on genetic assays of DMP, a total of 8 alleles were estimated at the sex-determining locus in *B. florilegus*, a species that has undergone a severe range contraction across its Japanese range (Takahashi *et al.*, 2008). The contrast between this value and the value estimated from the UK *B. hypnorum* population is again consistent with the lack of an extreme reduction in genetic diversity in the latter population.

Combining the genetic and productivity data in the *B. hypnorum* colonies reared from field-collected queens suggests that DMP decreased colony productivity (DMP colonies (n = 5): mean $n_{workers}$ = 18, mean n_{gynes} = 0, mean n_{males} = 15; non-DMP colonies (n = 32): mean $n_{workers}$ = 34, mean n_{gynes} = 6, mean n_{males} = 43). This suggestion is in line with previous findings in ants and bees (Ross & Fletcher, 1986; Whitehorn *et al.*, 2009; Gosterit, 2016; Bortolotti *et al.*, 2020), and exemplifies the fitness costs of matched matings in bumblebees. Such reductions in colony productivity may account for the lower DMP frequencies observed in mature, field-collected colonies than in colonies reared from field-collected queens (**Figure 2.3**), with smaller colonies being less likely to survive and be available for sampling, and support the assumption of sampling and/or survival bias in estimating DMP frequencies from field-collected colonies.

(c) Conclusions

In conclusion, genetic data from the UK *B. hypnorum* population showed relatively high genetic diversity, no evidence of a recent severe genetic bottleneck, and low levels of DMP, matching predictions from the gene flow hypothesis. Hence, colonisation of the UK by *B. hypnorum* does not represent an example of the genetic paradox of invasion (Jones & Brown, 2014; Schrieber & Lachmuth, 2017) or an example of a eusocial Hymenopteran achieving rapid range expansion despite high levels of DMP (Buttermore *et al.*, 1998; Darrouzet *et al.*, 2015). Alongside evidence of *B. hypnorum* undergoing a recent westward range expansion within Europe (Wagner, 1937; Rasmont, 1989; Rollin *et al.*, 2020), our findings suggest that this species may resemble other invertebrate taxa that have recently expanded their ranges at a continental scale. Examples in Europe include the Wasp Spider (*Argiope bruennichi*; Krehenwinkel & Tautz, 2013) and the Dainty Damselfly (*Coenagrion scitulum*; Swaegers *et al.*, 2013). In such cases, the central genetic phenomena are progressive loss of genetic diversity across an invasion front and its consequent impact on adaptability (Ibrahim *et al.*, 1996; García-Ramos & Rodríguez, 2002), along with the genetic processes, if any, that trigger range expansions away from the source population. Whether the above-mentioned genetic phenomena impact *B. hypnorum*'s distinctive pollinating role in the UK (Crowther *et al.*, 2014) remains to be discovered.

2.6. References

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Chapter 3

Host-parasite interactions between the Tree Bumblebee (*Bombus hypnorum*) and a generalist bumblebee nematode parasite (*Sphaerularia bombi*)

A sunbathing Tree Bumblebee (*Bombus hypnorum*) queen warms herself following emergence from hibernation.

CHAPTER 3

Host-parasite interactions between the Tree Bumblebee (*Bombus hypnorum*) and a generalist bumblebee nematode parasite (*Sphaerularia bombi*)

3.1. Abstract

Parasites are pervasive across ecosystems and, through the negative fitness impacts they inflict upon their hosts, can influence the population dynamics of host species. Bumblebees (*Bombus* spp.) host a broad community of generalist parasites, some of which have been linked to their widespread population declines. For this reason, there is special interest in understanding host-parasite interactions in bumblebees, and their potential influence on population trends. Here, I present the first comprehensive quantification of host-parasite interactions between the rapidly range-expanding Tree Bumblebee (*Bombus hypnorum*) and the generalist, castrating nematode parasite *Sphaerularia bombi*. I find that the UK *B. hypnorum* population exhibits a parasite prevalence and community structure similar to those of populations of native bumblebee species but exhibits some degree of resistance to the castrating effects of *S. bombi*. Nonetheless, *S. bombi* infection still has significant effects on queen fitness in *B. hypnorum*, with infected queens being less likely to successfully found colonies and exhibiting lower productivity and shorter longevity when compared to uninfected queens. Further, I show that *S. bombi* females rarely successfully reproduce inside the *B. hypnorum* host, with *S. bombi* larvae never being found in the host hindgut or faeces. Resistance to the castrating effects of *S. bombi* may be one factor underlying *B. hypnorum*'s rapid range expansion across the UK, while its representing an apparent 'dead-end' in the *S. bombi* life cycle may produce a parasite dilution effect, indirectly benefitting native UK bumblebee species.

3.2. Introduction

Given the significant ecological and economic threats posed by invasive species worldwide (Olson, 2006; Doherty *et al.*, 2016; Paini *et al.*, 2016), understanding the factors that influence the establishment and spread of a species in a new range represents a fundamental challenge in ecology. One potential explanation for successful range expansion is the enemy release hypothesis, which suggests that colonisation success is associated with the lack of natural enemies (i.e. competitors, predators and pathogens) encountered in the new range when compared to the native range (Jeffries & Lawton, 1984; Keane & Crawley, 2002; Mitchell & Power, 2003; Torchin *et al.*, 2003; Colautti *et al.*, 2004; Roy *et al.*, 2011). Accordingly, colonists may experience reduced population regulation in the new range, resulting in rapid ecological success (Colautti *et al.*, 2004; Roy *et al.*, 2011).

Parasites are pervasive across ecosystems, comprising an estimated 30-40% of all extant eukaryotes (De Meeûs & Renaud, 2002; Dobson *et al.*, 2008; Carlson *et al.*, 2020), and play key roles in influencing host species ecology and community interactions (Dobson & Hudson, 1986; Lefèvre *et al.*, 2009). Negative fitness impacts associated with parasitic infection are well reported (e.g. Torchin *et al.*, 2001; Fitze *et al.*, 2004), and there is both theoretical and empirical support for the role of parasites in regulating the abundance and density of host populations (e.g. Anderson, 1978; Anderson & May, 1981; Hudson *et al.*, 1998; Albon *et al.*, 2002). Parasites therefore represent one of the key components of the process of enemy release (Prenter *et al.*, 2004; Torchin & Mitchell, 2004), and there are numerous examples of invasive species experiencing reduced parasite loads in their new range when compared to the native range (e.g. Torchin *et al.*, 2003; Allen *et al.*, 2007; Sheath *et al.*, 2015; Schoeman *et al.*, 2019).

Bumblebees (*Bombus* spp.) are annual eusocial insects (**Figure 1.1**) that represent key pollinators of various wild plants and economically important crops worldwide (Klein *et al.*, 2007). Worryingly, these roles are threatened by widespread bumblebee population declines (Williams *et al.*, 2009; Cameron *et al.*, 2011; Arbetman *et al.*, 2017) that are linked to a combination of interacting factors including habitat loss, pesticide usage, and parasites (Goulson *et al.*, 2015). However, at regional scales, some bumblebees are expanding their ranges, either through human introduction (e.g. Semmens *et al.*, 1993; Matsumura *et al.*, 2003) or natural range-expansion processes (e.g. Martinet *et al.*, 2015; Biella *et al.*, 2020). Bumblebees host a broad community of parasites that impact both individual- and colony-level fitness traits and may influence population dynamics (Schmid-Hempel, 2001; Otterstatter & Whidden, 2004; Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2008; Gillespie, 2010). This lends a special interest to studying the impacts of parasites on bumblebee population trends (Macfarlane & Griffin, 1990; Gillespie, 2010; Cameron *et al.*, 2011; Arbetman *et al.*, 2017), including investigations of the potential role of the enemy release hypothesis in explaining successful bumblebee range-expansion (Allen *et al.*, 2007; Schmid-Hempel *et al.*, 2013; Jones & Brown, 2014).

The Tree Bumblebee (*B. hypnorum*) represents an ecologically successful bumblebee species that is currently undergoing a westward range expansion within Europe. Historically, *B. hypnorum*'s native range extends across continental Europe and Asia, with data pointing to increases in abundance across Germany and Belgium during the 20th century (Wagner, 1937; Rasmont, 1989), before expansions in the range of the species across the UK and Iceland from 2001 and 2008 onwards, respectively (Goulson & Williams, 2001; Prŷs-Jones *et al.*, 2016). Following its arrival in the UK, *B. hypnorum* has rapidly increased in range and abundance to become one of the most common and widespread UK bumblebee species (Crowther *et al.*, 2014, 2019; Jones & Brown, 2014; Goulson *et al.*, 2018), having undergone a range expansion of ~900 km in the 20 years since it was first recorded (**Figure 1.2**). Hence the colonisation of the UK by *B. hypnorum* represents an informative case-study to test parasite-driven factors that influence a species' ecological success.

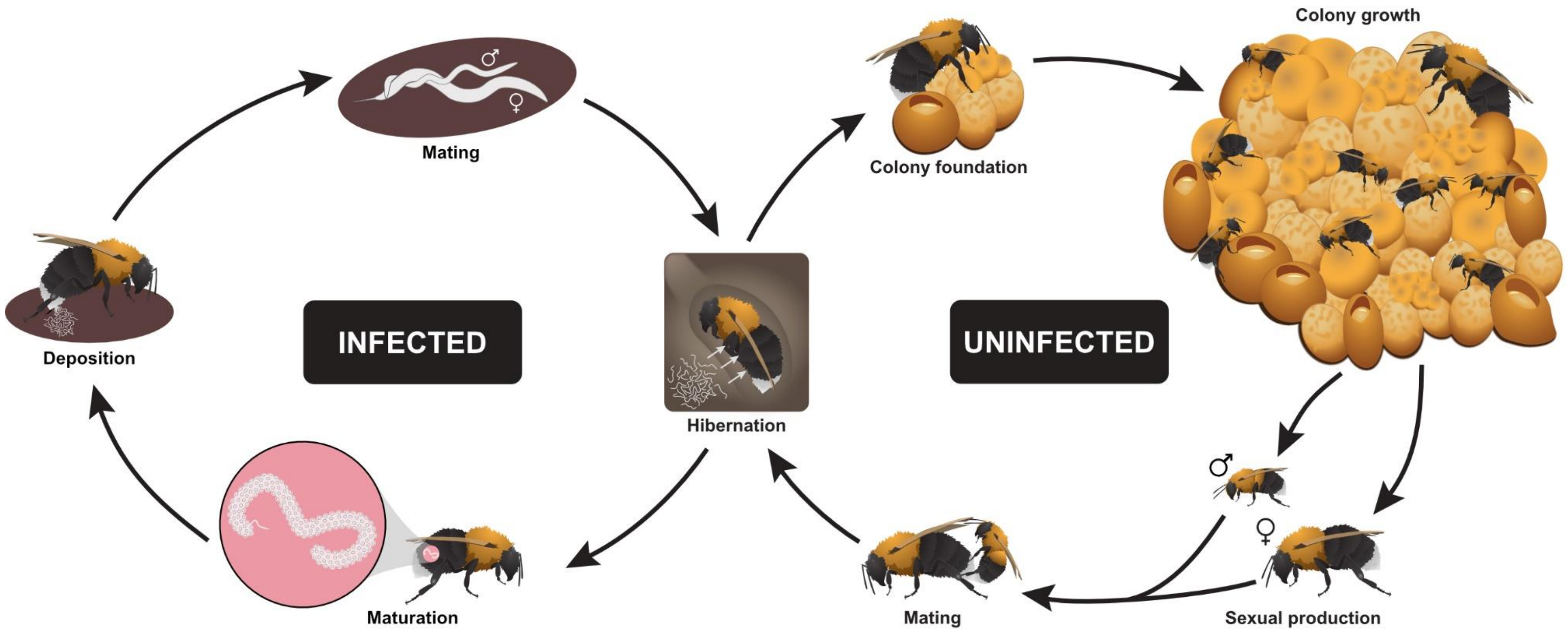

Figure 3.1. Host-parasite interactions between the nematode *Sphaerularia bombi* and its bumblebee (*Bombus* spp.) host. Bumblebee queens are infected during hibernation, with mated *S. bombi* females entering the host haemocoel and overwintering inside the queen. Once the infected queen (left) emerges from hibernation, the nematode matures and begins to produce eggs that, upon hatching, enter the host's digestive tract. Infection with *S. bombi* triggers behavioural changes in the host, with infected queens visiting overwintering sites and depositing juvenile nematodes, rather than founding colonies. The nematodes sexually mature in the soil and mate before the females encounter the next generation of hibernating queens. Uninfected queens (right) emerge from hibernation and carry out the bumblebee life cycle as normal.

A previous study demonstrated that UK *B. hypnorum* queens exhibit a parasite prevalence and community structure similar to those of five other native UK bumblebee species (*B. jonellus*, *B. lucorum*, *B. pascuorum*, *B. pratorum* and *B. terrestris*), suggesting that the successful colonisation of the UK by *B. hypnorum* was not influenced by enemy release (Jones & Brown, 2014). However, while *B. hypnorum* has not escaped from parasites in its newly-colonised range, other potential parasite-mediated mechanisms may explain its ecological success as a range-expanding bumblebee. Of particular interest is *B. hypnorum*'s potential resistance to the negative fitness impacts associated with infection by *Sphaerularia bombi* (Hasselrot, 1960; Röseler, 2002; Jones & Brown, 2014), a generalist bumblebee nematode parasite with a broad geographic range matching that of the *Bombus* genus (Poinar Jr & Van der Laan, 1972; Lundberg & Svensson, 1975; Macfarlane & Griffin, 1990; McCorquodale *et al.*, 1998; Kosaka *et al.*, 2008; Maxfield-Taylor *et al.*, 2011; Plischuk & Lange, 2012; Jones & Brown, 2014; Chauhan *et al.*, 2018).

Infection of *Bombus* queens by *S. bombi* occurs during hibernation, during which they encounter mated, infective stage *S. bombi* females that enter queens and migrate to their haemocoels (**Figure 3.1**; Poinar Jr & Van der Laan, 1972). On successful infection, *S. bombi* females begin to reproductively mature through the eversion and enlargement of the reproductive tract (**Figure 3.1**; **Figure 3.2E**; Poinar Jr & Van der Laan, 1972), with invaginations on the everted uterus allowing *S. bombi* to obtain resources directly from the host haemolymph (Poinar Jr & Hess, 1972). Upon the queen's emergence from hibernation, the *S. bombi* female completes reproductive maturation, with the uterus expanding to a size of up to 100 times that of the nematode's body, and begins producing eggs containing larval stage 1 (L1) juveniles (Poinar Jr & Van der Laan, 1972). The L1 juveniles undergo two moults inside the egg before eclosing as L3 juveniles, which then migrate to the host's digestive tract (Poinar Jr & Van der Laan, 1972). *S. bombi* infection leads to drastic behavioural changes in bumblebee queens, which, instead of founding colonies, begin visiting potential hibernation sites and depositing L3 *S. bombi* juveniles in the faeces (**Figure 3.1**; Poinar Jr & Van der Laan, 1972; Lundberg & Svensson, 1975). Following deposition, the L3 juveniles undergo two further moults to reach sexual maturity and mate, after which the mated *S. bombi* female remains in the hibernation site until she comes into contact with the next generation of overwintering bumblebee queens, starting the life-cycle again (**Figure 3.1**; Poinar Jr & Van der Laan, 1972).

S. bombi infection is associated with developmental reduction of the host queen's corpora allata, an endocrine organ that produces hormones essential for ovarian activation (Palm, 1948). Hence, the physiological and behavioural changes associated with *S. bombi* infection exert a significant cost on the host queen in the form of impairing the function of the gonads. Such effects likely represent a parasite adaptation to ensure successful completion of the life-cycle (Poulin, 1995), with host castration (i.e. the prevention of reproduction of both male and female hosts) representing a common strategy across parasite species (Baudoin, 1975; Lafferty & Kuris, 2009). Given its castrating effects, *S. bombi* is generally considered a 'high impact' bumblebee parasite species (Rutrecht & Brown, 2008; Jones & Brown, 2014), with evidence to date suggesting that *S. bombi* infection negatively impacts key life-history traits across bumblebee species in general (Rutrecht & Brown, 2008; Kelly, 2009). For instance, *S. bombi* infection causes complete castration in *B. pratorum*, with infected queens incapable of laying eggs (Rutrecht & Brown, 2008). Further, in both *B. lucorum* and *B. terrestris*, *S. bombi* infection leads to reduced ovarian activation and queen longevity, with both species unable to produce offspring when infected (Kelly, 2009; Jones & Brown, 2014). However, while fitness impacts of *S. bombi* infection are relatively well studied in some widespread and common bumblebee species, impacts in other species are mostly unknown and host castration by *S. bombi* may not be the rule across bumblebees.

Consistent with *S. bombi* having potentially variable effects across bumblebees, there is some anecdotal evidence to suggest that *B. hypnorum* exhibits resistance to the castrating effects of *S. bombi* infection (Hasselrot, 1960; Röseler, 2002; Jones & Brown, 2014). For instance, Hasselrot (1960) recorded successful egg laying in 2 of 4 infected queens from a Swedish population, Jones & Brown (2014) recorded egg-laying and successful offspring production in 5 and 2, respectively, of 17 infected queens, while Röseler (2002) recorded two infected queens that successfully founded colonies and produced both males and gynes. Therefore, I propose that resistance to the castrating impacts of *S. bombi* may represent a factor underlying the rapid colonisation and subsequent ecological success of *B. hypnorum* in the UK (**Figure 1.2**). Further, *B. hypnorum*'s potential resistance to *S. bombi* may impose significant fitness costs on the nematode itself, through leading the nematode to fail either to reproduce in the host or to transmit infective stages, making *B. hypnorum* a 'dead-end' host and inhibiting completion of the parasite lifecycle (**Figure 3.1**). However, quantitative studies detailing the host-parasite interactions between *B. hypnorum* and *S. bombi* are lacking to date.

Hence, the overall goal of the current study was to provide insight into host-parasite interactions between *B. hypnorum* and *S. bombi* within the UK *B. hypnorum* population, via three specific aims. Firstly, I sought to determine the prevalence and community structure of four generalist bumblebee parasite species (including *S. bombi*) in the UK *B. hypnorum* population, allowing comparison with the findings of Jones & Brown (2014). Secondly, I sought to quantify the fitness impacts associated with *S. bombi* infection in *B. hypnorum* queens. Finally, I sought to quantify the reproductive output of *S. bombi* females inside *B. hypnorum* queens, and thereby test whether *B. hypnorum* represents a suitable host for completion of the nematode life cycle. Based on previous observations (Röseler, 2002; Jones & Brown, 2014), I hypothesised that the study UK *B. hypnorum* population would exhibit a parasite prevalence and community structure similar to those of other UK bumblebee species and that queens exhibit some degree of resistance to the castrating effects of *S. bombi*. Further, given the potential resistance to *S. bombi* castration (Hasselrot, 1960; Röseler, 2002; Jones & Brown, 2014), I hypothesised that *B. hypnorum* would not represent a suitable host for the completion of the *S. bombi* lifecycle, with *S. bombi* females failing to reproduce in their *B. hypnorum* hosts.

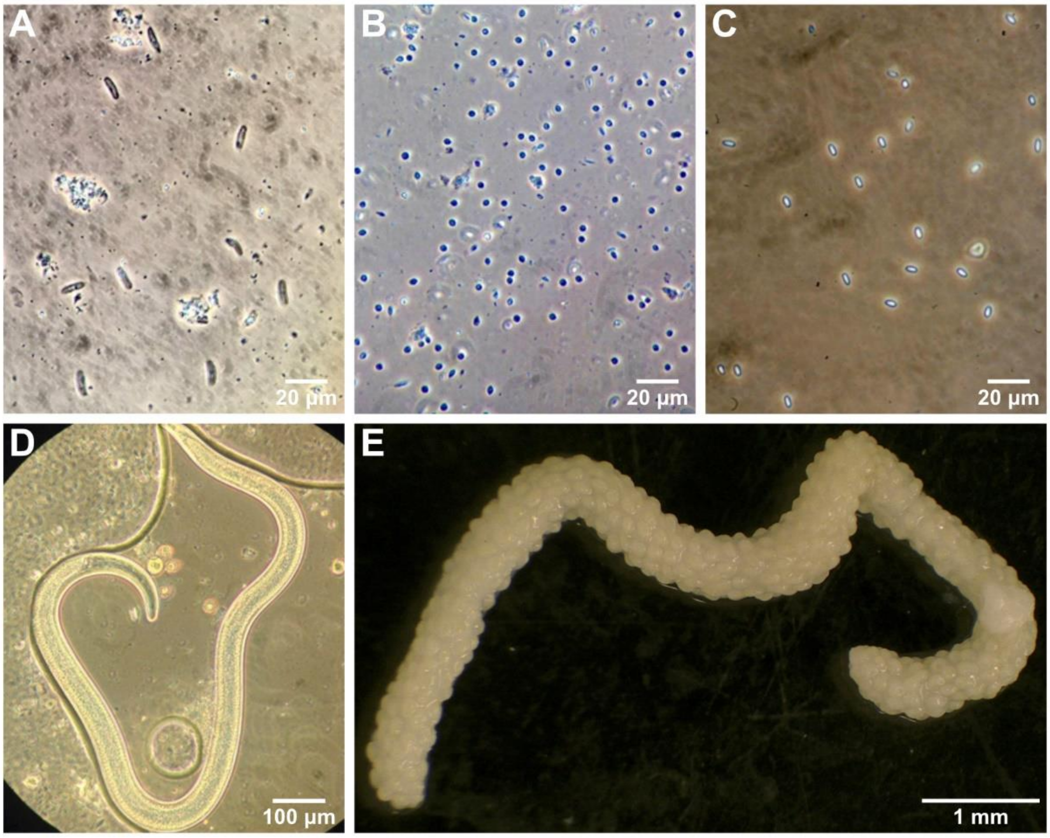

Figure 3.2. The four major endoparasite species of the bumblebee parasite community in the UK. **(A)** *Apicystis bombi*; **(B)** *Crithidia bombi*; **(C)** *Nosema bombi*; and **(D)** *Sphaerularia bombi* larva found in bumblebee queen faecal samples at 100-400× magnification. **(E)** The everted, enlarged uterus of a mature *S. bombi* female, dissected out from the abdomen of a Tree Bumblebee (*Bombus hypnorum*) queen. Note that, since *S. bombi* larvae were not present in the faeces of infected *B. hypnorum* queens (see **Results**), the *S. bombi* larva in image **D** is from the faeces of an infected *B. terrestris* queen. Images **A-D** taken using a GXCam HiChrome-Met (GTVision Ltd, Stansfield, UK) attached to an Olympus BX41 phase-contrast microscope (Olympus Corp., Tokyo, Japan), and used with permission of Dr Arran Folly. Image **E** taken using a GXCam HiChrome-S microscope camera (GTVision Ltd, Stansfield, UK) attached to a Leica M80 stereomicroscope (Leica Microsystems, Milton Keynes, UK).

3.3. Methods

(a) Queen collection and faecal parasite screening

Two cohorts of *B. hypnorum* queens (*n* = 263) were collected from the field between 7 March and 19 April 2018 and between 25 February and 9 April 2019, respectively. Queens were captured with an entomological net while they were foraging or nest-searching and were then transferred to individual plastic vials in a chilled container before transportation to the laboratory. The queens in the 2018 cohort (*n* = 107) were collected from Great Windsor Park, Surrey (*n* = 25), Chingford, Greater London (*n* = 7), and various sites within a 5 km radius from the city centre of Norwich, Norfolk (*n* = 75). All queens in the 2019 cohort (*n* = 156) were collected in Norwich. Queens bearing pollen on their corbiculae were not collected, since such queens are likely to have initiated nesting and to be gathering pollen to provision their brood (Alford, 2011). Further, queens that have already initiated nests in the field may be less likely to found nests under captive conditions (Tripodi & Strange, 2019). Given the lack of evidence for genetic differentiation between subpopulations of *B. hypnorum* across the UK (Huml *et al.*, 2021), queens collected across all sites were pooled in the present study, and considered representative of the UK population.

Infection status of the collected *B. hypnorum* queens was determined by screening faecal samples under an Olympus BX41 phase-contrast microscope (Olympus Corp., Tokyo, Japan) at 100-400× magnification. Approximately 10μl of queen faeces were screened for four generalist bumblebee endoparasites that each have at least one life stage occurring in bee faeces and that are readily identifiable using phase-contrast microscopy (Rutrecht & Brown, 2008): (1) the apicomplexan *Apicystis bombi* (Neogregarinorida: Lipotrophidae; **Figure 3.2A**); (2) the trypanosome *Crithidia bombi* (Trypanosomatida: Trypanosomatidae; **Figure 3.2B**); (3) the microsporidian *Nosema bombi* (Dissociohaplophasida: Nosematidae; **Figure 3.2C**); and (4) the nematode *Sphaerularia bombi* (Tylenchida: Sphaerulariidae; **Figure**

3.2D). These four parasite species were considered since: (1) they represent the most abundant and well-studied endoparasites in the bumblebee literature (e.g. Schmid-Hempel, 2001; Rutrecht & Brown, 2008); and (2) it allowed direct comparisons with the findings of Jones & Brown (2014).

S. bombi-infected bumblebee queens begin shedding nematode larvae in the faeces two weeks after the queens' emergence from hibernation, suggesting that *S. bombi* females need fewer than two weeks' time to begin producing eggs (Kelly, 2009). Accordingly, each queen from the 2018 cohort was screened within 24 hours of collection, and once again 7-10 days post-capture. Given that no *S. bombi* larvae were found in the faeces of the 2018 queen cohort (see **Results**), the period between the first and second parasite screenings in the 2019 queen cohort was extended. Accordingly, each queen from the 2019 cohort was screened within 24 hours of collection, and once again 21 days post-capture, ensuring that if queens were infected with *S. bombi* then the infected queens should be shedding *S. bombi* larvae in the faeces by this time (Kelly, 2009). Queens that died before the first parasite screening were excluded from the study. For queens that died between the first and the second parasite screening, the hindgut was dissected from each queen, pulverized using a pestle and mortar, and approximately 10μl of the homogenate was screened for the presence of the above parasites (**Figure 3.2A-D**) at 100-400× under a phase-contrast microscope.

(b) Queen and colony rearing

Queens were reared in custom-made acrylic queen-rearing boxes (dimensions: L14 × W8 × D5 cm; Signforce Ltd, Norwich, UK) and kept at constant conditions (mean ± range: 27°C ± 1°C; 60% ± 10% RH), with pollen (Sussex Wholefoods, Eastbourne, UK) and 50% Apiinvert sugar syrup (Südzucker AG, Mannheim, Germany) provided *ad libitum*. Rearing boxes were checked daily for the presence of queen-laid eggs/larvae in 2019, allowing the confirmation of whether each *B. hypnorum* queen had successfully laid eggs. Rearing boxes were checked daily for adult offspring (i.e. workers or males) production in both 2018 and 2019, allowing the confirmation of whether each *B. hypnorum* queen had successfully produced offspring. On the eclosion (adult emergence from pupa) of 10-20 workers, the queen, workers, and brood (eggs, larvae, and pupae) were transferred to wooden nest-boxes (dimensions: L30 × W20 × D17 cm), with pollen and sugar syrup continuing to be provided *ad libitum*. All adult workers were individually marked with numbered discs (EH Thorne Ltd, Market Rasen, UK) upon eclosion. All adult sexuals (males and gynes) were removed and frozen at -20˚C upon eclosion, since, in wild bumblebee colonies, sexuals disperse from the colony a few days after eclosion (Alford, 2011). Queens/colonies were monitored daily for dead individuals (colony queen or workers), which, once their individual identities and date of death had been recorded, were removed from the colony and frozen at -20˚C. Daily monitoring of the reared queens/colonies therefore allowed the collection of demographic data for each queen/colony.

To ensure parasite cross-contamination did not occur between queens, infected queens were kept separately from uninfected queens, and separate sets of handling equipment (e.g. tongs) were used for the two classes. Infected queens were defined as those whose faeces were found to contain one or more of the four parasite species checked for during any of the faecal screens (**Figure 3.2A-D**), and uninfected queens were defined as those whose faeces were not found to contain any of the four parasite species during either of the faecal screens. Further potential for cross-contamination was reduced by the sterilisation of handling equipment between the handling of queens/colonies within both infected and uninfected groups. Besides this, infected queens and their colonies were reared and handled identically to uninfected queens and colonies.

S. bombi larvae were never found during faecal screening (see **Results**) and *S. bombi* infection was therefore verified by the post-death dissection of queen abdomens to check for the presence of *S. bombi* (as detailed in the next section). Given that *S. bombi* infection could not be determined by faecal screening, *S. bombi*-infected queens would have initially been falsely considered as uninfected during queen rearing and reared alongside other genuinely uninfected queens. However, given the sterilisation of handling equipment in between colony handling, and that bumblebee queens are only vulnerable to *S. bombi* infection when they encounter the mated, infective-stage females during hibernation (**Figure 3.1**; Poinar Jr & Van der Laan, 1972), there would have been no chance of *S. bombi* cross-infection between queens.

(c) Queen dissections and *Sphaerularia* assessment

Following death, the abdomen of each queen was dissected at 10-30× magnification under a Zeiss Discovery v12 stereomicroscope (Zeiss, Oberkochen, Germany) to check for the presence of mature adult *S. bombi* females, defined as those nematodes that have successfully infected the host queen and everted their uterus (**Figure 3.2E**; **Figure 3.3**). The number of *S. bombi* uteri within the abdomen was recorded for each infected queen (henceforth described as infection intensity), with each uterus individually removed from the queen abdomen and photographed using a Zeiss AxioCam MCr5 microscope camera (Zeiss, Oberkochen, Germany). The length (in mm) of each *S. bombi* uterus was measured using the freeform line tool in the Fiji-ImageJ software suite (Schindelin *et al.*, 2012).

On the confirmation of *S. bombi* infection, the hindgut was removed from each infected queen and pulverized using a pestle and mortar. A 10μl sample of the infected queen's hindgut and haemocoel were then screened at 100-400× under a phase-contrast microscope, to check for the presence of *S. bombi* eggs and larvae in the haemocoel, and *S. bombi* larvae in the hindgut. Screening of the haemocoel and hindgut contents therefore allowed the confirmation of (1) whether *S. bombi* females were successfully reproducing in the host, and (2) whether *S. bombi* larvae were successfully passing from the haemocoel into the gut ready to be transmitted in the faeces.

Figure 3.3. The dissected abdomen of a Tree Bumblebee (*Bombus hypnorum*) queen infected with the obligately parasitic nematode worm *Sphaerularia bombi*. Each white, translucent 'worm' is the everted and enlarged uterus of a mature *S. bombi* female, and hence this queen is infected by multiple individuals. Image taken using a Zeiss AxioCam MCr5 (Zeiss, Oberkocken, Germany) attached to a Zeiss Discovery v12 stereomicroscope.

(d) *Sphaerularia* impacts on queen fitness

Three different metrics were used to measure queen fitness across *S. bombi*-infected and *S. bombi*-uninfected queens (defined as those queens that exhibited no evidence of *S. bombi*-infection following both faecal/hindgut screening and abdominal dissection). Firstly, queen fitness was measured as the percentage of queens successfully reaching the following milestones, all of which are essential for successful completion of the colony cycle in eusocial species: (1) egg-laying, defined as whether queens laid at least one egg (note that this measure was only available for the 2019 queen cohort); (2) adult offspring production, defined as the successful production of at least one adult offspring; and (3) colony foundation, defined as the successful production of 5 or more adult workers by the queen. Percentage rates for reaching each milestone were calculated by dividing the number of successful queens by the total number of queens. Secondly, for those queens that successfully produced adult offspring, queen fitness was measured as the numeric total of workers, males, gynes, and sexuals (males plus gynes) produced by each queen. Finally, given queen longevity is positively associated with reproductive success in several eusocial species, including bumblebees (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015; **Chapter 4**), queen longevity, defined as the time in days between queen capture and queen death, was also measured as an index of fitness. This represented a crude measure of queen longevity, since the eclosion date of each queen, and hence their true longevity, was unknown due to the queens having been field-collected. However, this measure of longevity can still provide insight into the impacts of *S. bombi* infection on queen fitness, given that it provides a metric of queen longevity post-hibernation, when queens begin to produce offspring and gain fitness. Impacts of *S. bombi* infection on all queen fitness measures were tested using univariate analyses between uninfected and *S. bombi*-infected queens (as detailed in the next section).

(e) Statistical analyses

All statistical analyses were carried out using R v4.0.1 (R Core Team, 2020), and all data were visualised with the *ggplot2* R package (Wickham, 2009).

Fisher's exact tests were used to test differences in the prevalence of each parasite species between the two queen cohorts. A two-sample t-test was used to test differences in uterus length between *S. bombi* females found across both years and a two-sample Wilcoxon test was used to test differences in *S. bombi* infection intensity between years. Relationships between *S. bombi* uterus length and infection intensity (i.e. whether *S. bombi* females were smaller at higher infection intensities) and queen longevity and infection intensity (i.e. whether queens suffering from higher infection intensities were shorter lived) were analysed with general linear models.

Finally, Fisher's exact tests were used to test differences in the reproductive success of *S. bombi* females between years.

All analyses investigating *S. bombi* impacts on queen fitness were univariate, with the queen infection status (uninfected vs. *S. bombi*-infected) used as the predictor variable. Fisher's exact tests were used to test differences between the number of uninfected and *S. bombi*-infected queens that successfully laid eggs, produced adult offspring, and founded colonies. To determine the impact of *S. bombi* infection on the total number of workers, males, gynes, and sexuals produced by each offspring-producing queen, four generalised linear models (one for each response variable) with negative binomial distributions were fitted using the *glm.nb()* function from the *MASS* R package (Venables & Ripley, 2002). Finally, to test the impact of *S. bombi* infection on queen longevity, Kaplan-Meier survival curves and log-rank tests were carried out using the *survival* R package (Therneau, 2020).

Assumptions for all constructed models were visually assessed using the *autoplot()* function from the *ggfortify* R package (Tang *et al.*, 2016). All means are presented ± 1 standard deviation unless otherwise stated.

3.4. Results

(a) Parasite prevalence and community structure

Three queens from the 2018 cohort died before their first parasite screening. Hence, infection data were available for a total of 260 (98.9%) of the 263 field-collected queens ($n_{2018} = 104$; $n_{2019} = 156$).

Of the 2018 cohort, 69 queens (66.4%) were uninfected, 31 (28.8%) were infected with a single parasite species, and 4 (3.9%) were infected with two parasite species (defined as a dual infection; **Figure 3.4**). In 2018, *A. bombi* occurred at a prevalence of 1% (i.e. detected in 1 of the 104 screened and dissected queens), *C. bombi* at a prevalence of 22.2% (22/104), *N. bombi* at a prevalence of 1% (1/104), and *S. bombi* at a prevalence of 14.4% (15/104;

Figure 3.4A). Of the 31 single infections found in the 2018 cohort, 18 queens (17.3%) were infected with *C. bombi*, 1 queen (1%) was infected with *N. bombi*, and 12 queens (11.5%) were infected with *S. bombi* (**Figure 3.4B**). Of the four dual infections in the 2018 cohort, 1 queen (1%) was infected with both *A. bombi* and *C. bombi* and 3 queens (2.9%) were infected with both *C. bombi* and *S. bombi* (**Figure 3.4B**).

Of the 2019 cohort, 94 queens (60.3%) were uninfected, 48 (30.8%) were infected with a single parasite species, and 14 (9%) had a dual infection (**Figure 3.4**). In 2019, *A. bombi* occurred at a prevalence of 4.5% (7/156), *C. bombi* at a prevalence of 24.4% (38/156), *N. bombi* at a prevalence of 1.9% (3/156) and *S. bombi* at a prevalence of 17.9% (28/156; **Figure 3.4A**). Of the 48 single infections found in the 2019 cohort, 4 queens (2.6%) were infected with *A. bombi*, 27 queens (17.3%) were infected with *C. bombi*, 2 queens (1.3%) were infected with *N. bombi*, and 15 queens (9.6%) were infected with *S. bombi* (**Figure 3.4C**). Of the 14 dual infections in the 2019 cohort, 1 queen (0.6%) was infected with both *A. bombi* and *C. bombi*, 2 queens (1.3%) were infected with both *A. bombi* and *S. bombi*, 10 queens (6.4%) were infected with both *C. bombi* and *S. bombi*, and 1 queen (0.6%) was infected with both *N. bombi* and *S. bombi* (**Figure 3.4C**).

There was no significant difference in prevalence for any of the parasite species across the two queen cohorts (Fisher's exact test: *A. bombi* $p = 0.14$; *C. bombi* $p = 0.65$; *N. bombi* $p = 0.65$; *S. bombi* $p = 0.50$). Therefore, combining the two cohorts, *A. bombi* occurred at a prevalence of 3.1% (4/260), *C. bombi* at a prevalence of 23.1% (60/260), *N. bombi* at a prevalence of 1.5% (4/260), and *S. bombi* at a prevalence of 16.5% (43/260) in the study population. No queens in either of the cohorts were infected with more than two of the four parasite species (**Figure 3.4B-C**).

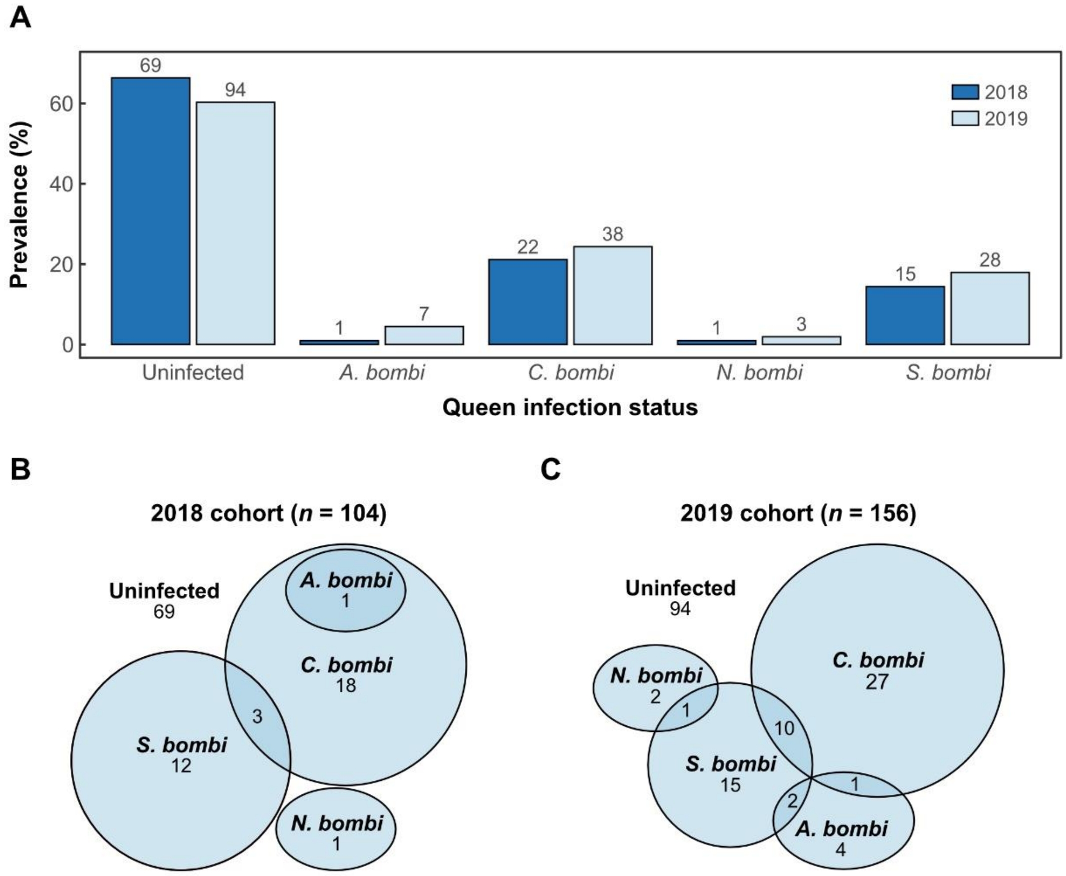

Figure 3.4. Parasite prevalence and community structure of four generalist bumblebee endoparasites across two cohorts of Tree Bumblebee (*Bombus hypnorum*) queens from a representative UK population. The parasite species are *Apicystis bombi*, *Crithidia bombi*, *Nosema bombi* and *Sphaerularia bombi*. **(A)** The percent prevalence of each parasite from queens collected in 2018 (dark blue bars; *n* = 104) and 2019 (light blue bars; *n* = 156). Prevalence was calculated for each parasite species by dividing the number of queens found to be infected by each parasite (numbers above each bar) by the total number of collected queens. Note that, due to dual parasite infections (i.e. a single queen infected by two different parasite species), numbers above the bars total to higher than the total number of queens collected. **(B)** Diagram of parasite community structure in the 2018 queen cohort. **(C)** Diagram of parasite community structure in the 2019 queen cohort. Numbers underneath each parasite species name represent the number of infected queens, with numbers within the overlapping areas indicating dual parasite infections. Note that the circle sizes in panels **(B)** and **(C)** are for illustrative purposes and are not directly proportional to the number of infected queens across the two cohorts.

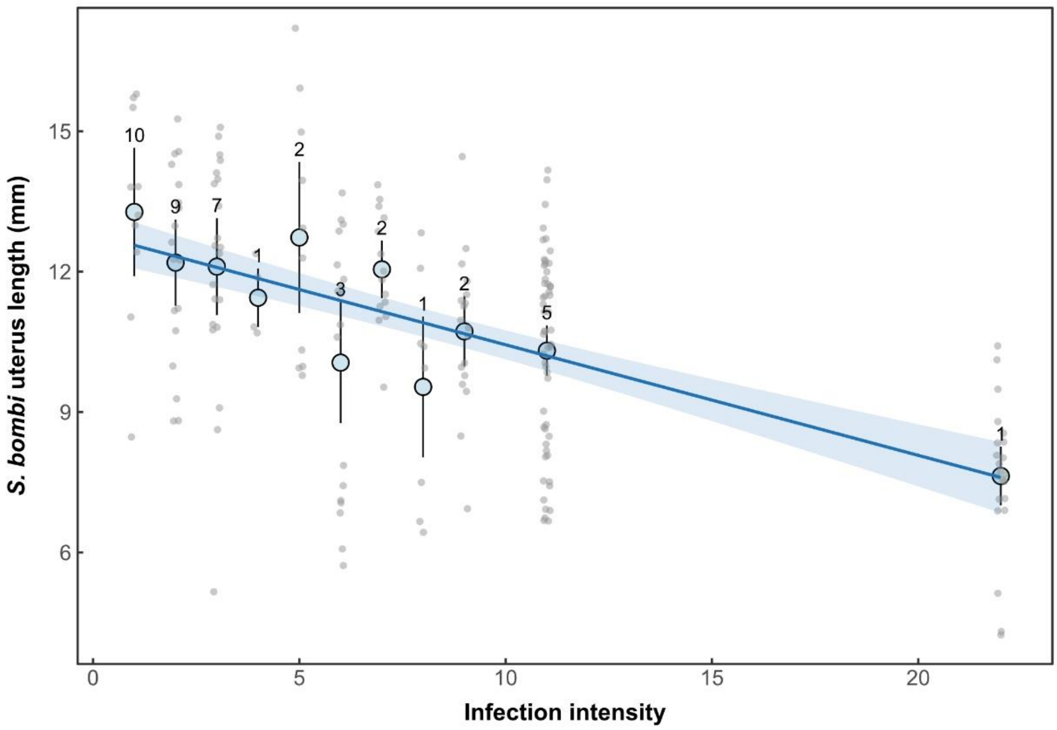

Figure 3.5. Uterus length (in mm) of each mature *Sphaerularia bombi* female as a function of infection intensity (i.e. the number of *S. bombi* females per host queen) across 43 infected Tree Bumblebee (*Bombus hypnorum*) queens. Navy line with shaded area, linear model (with 95% confidence interval) for uterus length by infection intensity ($F = 81.24$, $t = -9.01$, $p < 0.001$, Adj. $R^2 = 0.29$); filled blue circles with whiskers, mean ± 95% confidence interval uterus length at each infection intensity; numbers above whiskers, the number of queens at each infection intensity. Each grey point represents a single *S. bombi* female ($n = 198$) and, for illustrative purposes, each point is jittered to limit overlaps.

(b) *Sphaerularia* infection intensity

A total of 198 mature *S. bombi* females ($n_{2018} = 71$; $n_{2019} = 127$) were found in the abdomens of the 43 *S. bombi*-infected queens from both cohorts ($n_{2018} = 15$; $n_{2019} = 28$). Multiple infection by *S. bombi* was common, with 76.7% (33/43) of the infected queens exhibiting infection intensities of 2-22 *S. bombi* females and the remaining 23.3% (10/43) of the infected queens containing a single mature *S. bombi* female (**Figure 3.3**; **Figure 3.5**). There was no significant difference in infection intensity between the two cohorts (Wilcoxon rank sum test: W = 201.5, $p = 0.84$), with mean infection intensities of 4.7 ± 4.1 and 4.5 ± 4.5 *S. bombi* females per queen in the 2018 and 2019 cohorts, respectively.

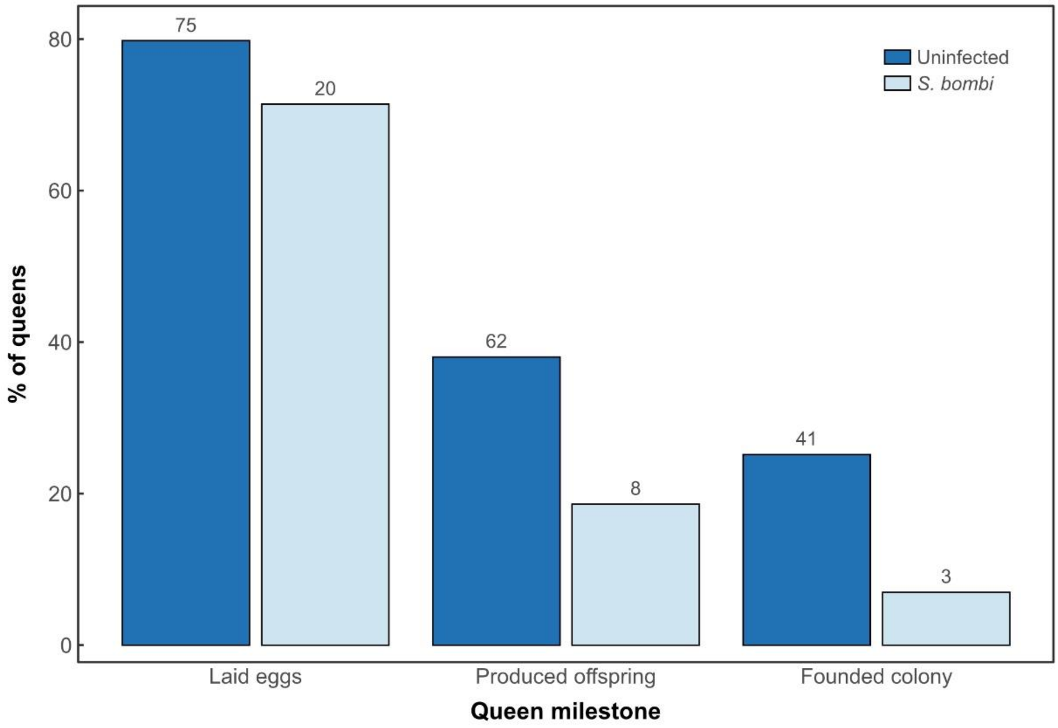

Figure 3.6. Percent success rates of egg laying, adult offspring production (defined as the rearing of at least one adult offspring) and colony foundation (defined as the rearing of 5 or more workers) by uninfected (dark blue bars) and *Sphaerularia bombi*-infected (light blue bars) Tree Bumblebee (*Bombus hypnorum*) queens. Numbers above each bar represent the number of successful queens at each life-cycle 'milestone'. Percentage success for each milestone was calculated by dividing the number of successful queens by the total number of queens for each group. Note that, since data for successful egg-laying were collected only for the 2019 queen cohort, the total queen sample sizes for each group are as follows: laid eggs, $n_{uninfected}$ = 94, $n_{S.\ bombi}$ = 28; produced offspring, $n_{uninfected}$ = 163, $n_{S.\ bombi}$ = 43; founded colony, $n_{uninfected}$ = 163, $n_{S.\ bombi}$ = 43.

All 198 *S. bombi* females had everted their uteri (and were hence considered reproductively mature), with a mean uterus length of 10.8 ± 2.5 mm. There was a significant difference in *S. bombi* uterus size between the two cohorts (Two-sample t-test: t = -2.62, p = 0.01), with mean uterus lengths of 10.2 ± 2.4 mm and 11.1 ± 2.6 mm in the 2018 and 2019 *S. bombi* females, respectively. There was a significantly negative relationship between infection intensity and *S. bombi* uterus length ($F = 81.2$, $t = -9$, $p < 0.001$; **Figure 3.5**), such that *S. bombi* uteri were larger in the presence of fewer conspecifics (i.e. lower infection intensity) in their *B. hypnorum* queen hosts.

(c) *Sphaerularia* impacts on queen fitness

There was no difference in successful egg-laying between uninfected and *S. bombi*-infected queens (Fisher's exact test: $p = 0.44$), with 79.8% (75/94) of uninfected queens and 71.4% (20/28) of *S. bombi*-infected queens laying eggs, respectively (**Figure 3.6**). However, uninfected queens were significantly more likely to successfully produce adult offspring than *S. bombi*-infected queens (Fisher's exact test: $p = 0.02$), with 38% (62/163) of uninfected queens and 18.6% (8/43) of *S. bombi*-infected queens managing to produce at least one adult offspring, respectively (**Figure 3.6**). Further, uninfected queens were also more likely to successfully found colonies than *S. bombi*-infected queens (Fisher's exact test: $p = 0.01$), with 25.2% (41/163) of uninfected queens and 6% (3/43) of *S. bombi*-infected queens managing to produce 5 or more workers, respectively (**Figure 3.6**).

Of the 70 queens that successfully produced adult offspring, 62 queens (88.6%) were uninfected and 8 queens (11.4%) were *S. bombi*-infected (**Figure 3.6**). Of these, 91.9% (57/62) of the uninfected queens and 75% (6/8) of the *S. bombi*-infected queens successfully produced at least one adult worker. Further, 79% (49/62) of the uninfected, adult offspring-producing queens and 75% (6/8) of the *S. bombi*-infected queens successfully produced at least one adult male. Finally, 46.8% (29/62) of the uninfected, adult offspring-producing queens and 12.5% (1/8) of the *S. bombi*-infected queens successfully produced at least one adult gyne. Overall, 79% (49/62) of the uninfected, adult offspring-producing queens produced at least one adult sexual, with 20 queens (32.3%) successfully producing adult males, and 29 queens (46.8%) successfully producing both adult males and gynes. Of the *S. bombi*-infected, adult offspring-producing queens, 75% (6/8) produced at least one adult sexual, with 5 queens (62.5%) successfully producing adult males, and 1 queen (12.5%) successfully producing both adult males and gynes. Hence, of all queens found to be infected with *S. bombi*, 13% (6/43) managed to successfully produce adult sexuals.

Table 3.1. Model summaries for the impact of *Sphaerularia bombi* infection on the total number of adult workers, males, gynes, and sexuals (males plus gynes) produced by Tree Bumblebee (*Bombus hypnorum*) queens. Term, name of specified fixed predictor ('Intercept' = uninfected queen, '*S. bombi*' = *S. bombi*-infected queen); Estimate, parameter estimate for each predictor; SE, standard error of estimate; *z*, Wald z-test statistic; *p*, p-value, with a value of <0.05 indicating that the null hypothesis of the predictor effect being zero can be rejected.

Term	Estimate	SE	*z*	*p*
Total workers				
Intercept	3.841	0.188	20.437	<0.001
S. bombi	-1.292	0.563	-2.294	0.022
Total males				
Intercept	4.484	0.247	18.127	<0.001
S. bombi	-0.585	0.733	-0.798	0.425
Total gynes				
Intercept	3.227	0.364	8.870	<0.001
S. bombi	-4.613	1.286	-3.588	<0.001
Total sexuals				
Intercept	4.734	0.254	18.654	<0.001
S. bombi	-0.830	0.752	-1.104	0.270

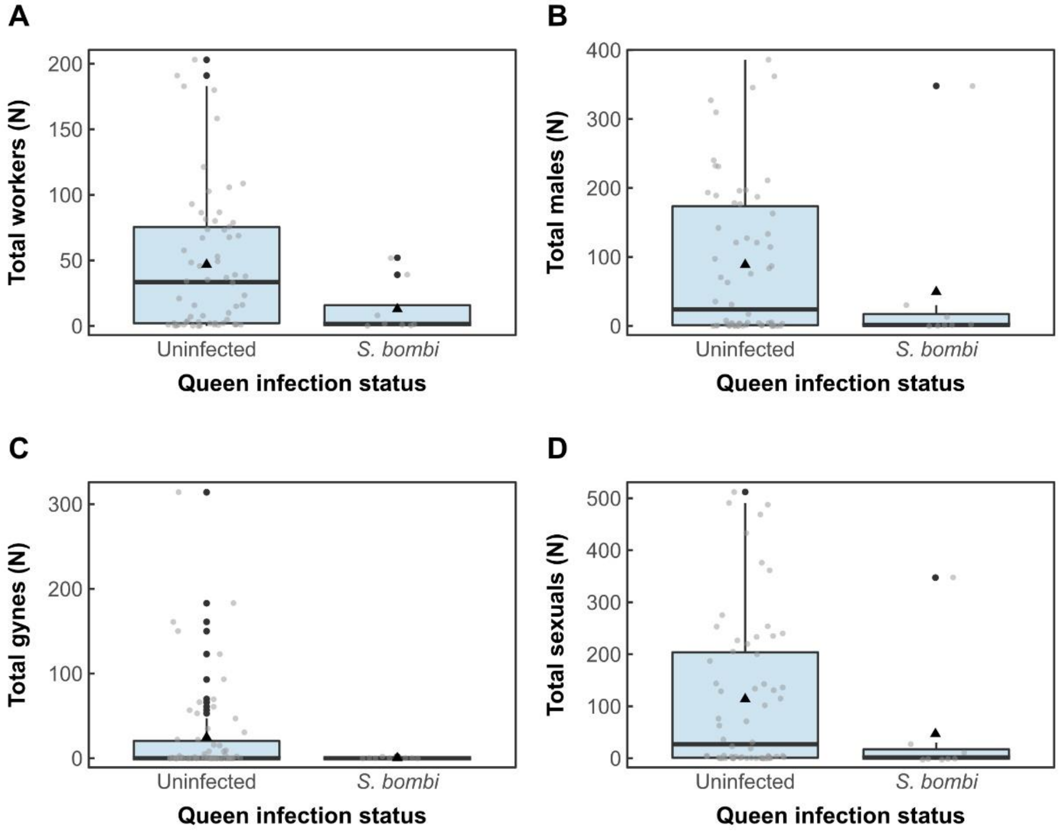

Figure 3.7. Productivity differences between uninfected (*n* = 62) and *Sphaerularia bombi*-infected (*n* = 8) Tree Bumblebee (*Bombus hypnorum*) queens that successfully produced at least one adult offspring. **(A)** total number of adult workers produced; **(B)** total number of adult males produced; **(C)** total number of adult gynes produced; and **(D)** total number of adult sexuals (males plus gynes) produced. Thick horizontal bars, median; triangles, mean; boxes, interquartile range (IQR); filled black circles, outliers, defined as points more than 1.5 IQR above or below the upper or lower quartile; filled grey circles, 'jittered' raw data, with each point representing a single offspring-producing queen.

Infection with *S. bombi* had a significantly negative impact on the number of adult workers produced by adult offspring-producing queens (z = -2.29, *p* = 0.02; **Table 3.1**), with uninfected and *S. bombi*-infected queens producing a mean of 46.9 ± 53.9 and 12.9 ± 20.6 workers, respectively (**Figure 3.7A**). *S. bombi* infection also reduced the number of adult males produced by adult offspring-producing queens, with uninfected and *S. bombi*-infected queens producing a mean of 88.6 ± 109 and 49.4 ± 121.1 males, respectively (**Figure 3.7B**), but this reduction was non-significant (z = -0.8, *p* = 0.43; **Table 3.1**). *S.*

bombi infection also significantly reduced the number of adult gynes produced by adult offspring-producing queens (z = -3.59, $p < 0.001$; **Table 3.1**), with uninfected and *S. bombi*-infected queens producing a mean of 25.2 ± 55.8 and 0.3 ± 0.7 gynes, respectively (**Figure 3.7C**). Finally, *S. bombi* infection reduced the total number of sexuals produced by adult offspring-producing queens, with a mean of 113.8 ± 149.3 and 49.6 ± 121.8 sexuals produced by uninfected and *S. bombi*-infected queens, respectively (**Figure 3.7D**), but this reduction was non-significant (z = -1.1, $p = 0.27$; **Table 3.1**).

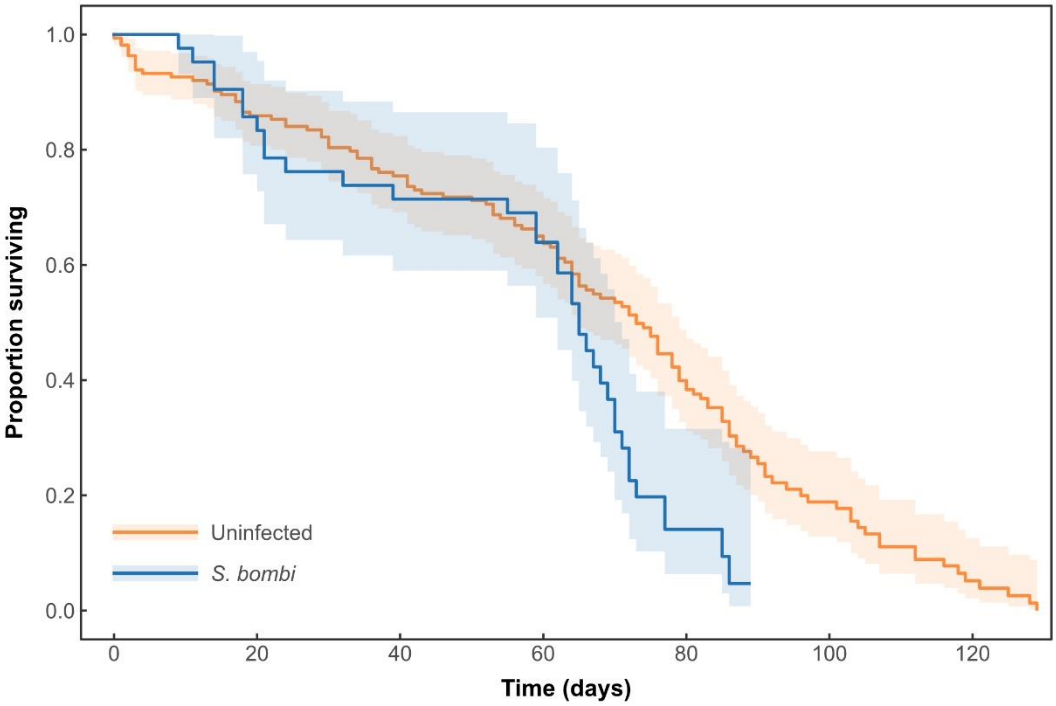

Figure 3.8. Percent survivorship with 95% confidence intervals of uninfected (orange line; *n* = 163) and *Sphaerularia bombi*-infected (blue line; *n* = 42) Tree Bumblebee (*Bombus hypnorum*) queens, i.e. proportion alive as a function of time in days since queen collection.

Longevity could be calculated for all uninfected queens and for 97.7% (42/43) of the *S. bombi*-infected queens. Uninfected queens were found to live significantly longer than *S. bombi*-infected queens (Log rank test: $\chi^2 = 7.5$, $p = 0.006$) with median longevities of 73 and 65 days, respectively (**Figure 3.8**). There was no relationship between the longevity of *S. bombi*-infected queens and infection intensity ($F = 0.1$, $t = 0.3$, $p = 0.76$; **Figure 3.9**), such that queens with low infection intensity were not significantly longer-lived than queens with high infection intensity.

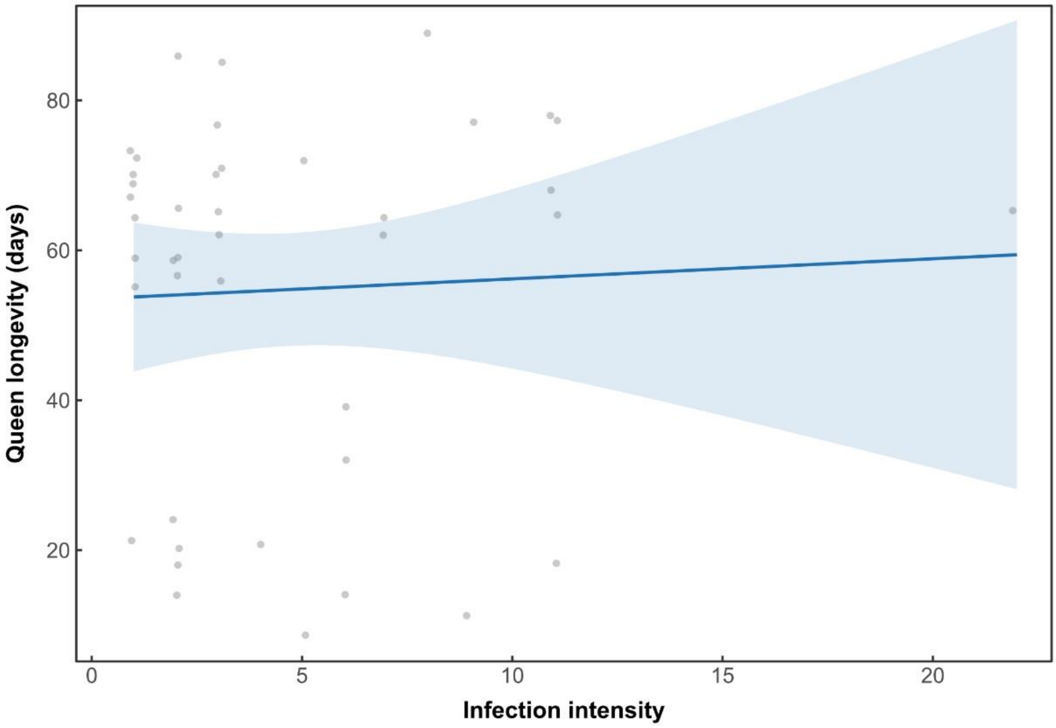

Figure 3.9. Longevity (in days) of Tree Bumblebee (*Bombus hypnorum*) queens infected with the nematode *Sphaerularia bombi* as a function of infection intensity (i.e. the number of *S. bombi* females per host queen). Queen longevity was defined as the time in days between queen collection and queen death. Navy line with shaded area, linear model (with 95% confidence interval) for longevity by infection intensity ($F = 0.10$, $t = 0.31$, $p = 0.76$, Adj. $R^2 = -0.02$). Each grey point represents a single *S. bombi*-infected queen ($n = 42$) and, for illustrative purposes, each point is jittered to limit overlaps.

(d) *Sphaerularia* reproductive success

None of the 43 *S. bombi*-infected queens deposited *S. bombi* larvae in their faeces (**Table 3.2**). Haemocoel screening of the infected queens revealed that *S. bombi* eggs and larvae were only present in the haemocoel of 16.3% (7/43) and 4.7% (2/43) of all infected queens, respectively (**Table 3.2**). There was no difference in the presence of *S. bombi* eggs in the haemocoel across the two cohorts (Fisher's exact test: $p = 1$), with *S. bombi* eggs being found in the haemocoel of 13.3% (2/15) and 17.9% (5/28) of the 2018 and 2019 queens, respectively (**Table 3.2**). Further, no difference was found in the presence of *S. bombi* larvae in the queen haemocoel across the two cohorts (Fisher's exact test: *p* = 1), with *S. bombi* larvae being found in the haemocoel of 6.7% (1/15) and 3.1% (1/28) of the 2018 and 2019 queens, respectively (**Table 3.2**). Finally, *S. bombi* larvae were never found in the hindgut of any infected queens (**Table 3.2**).

Table 3.2. Reproductive output of *Sphaerularia bombi* females, defined as the presence of *S. bombi* eggs or larvae within the faeces, haemocoel, or gut of their Tree Bumblebee (*Bombus hypnorum*) queen hosts. Year, the rearing year (2018 or 2019) for the *S. bombi*-infected queens, with numbers in parentheses underneath each year representing the number of *S. bombi*-infected queens within each cohort; Present/Absent, the presence or absence of *S. bombi* juvenile stages (eggs or larvae) in the infected queens; Faeces, the number of infected queens that shed *S. bombi* larvae in the faeces; Haemocoel, the number of infected queens that contained *S. bombi* eggs or larvae in the haemocoel; Gut, the number of infected queens that contained *S. bombi* larvae in the hindgut.

		Faeces	**Haemocoel**		**Gut**
Year	**Present/absent**	Larvae	Eggs	Larvae	Larvae
2018	Present	0	2	1	0
(*n* = 15)	Absent	15	13	14	15
2019	Present	0	5	1	0
(*n* = 28)	Absent	28	23	27	28
Total	Present	0	7	2	0
(*n* = 43)	Absent	43	36	41	43

3.5. Discussion

This study presents the first quantitative characterisation of host-parasite interactions between the rapidly range-expanding and ecologically successful

Tree Bumblebee (*Bombus hypnorum*) and the generalist bumblebee nematode parasite *Sphaerularia bombi*, representing one of few quantitative analyses of host-parasite interactions in a range-expanding bumblebee (Allen *et al.*, 2007; Schmid-Hempel *et al.*, 2013; Jones & Brown, 2014). Interestingly, while *B. hypnorum* queens were susceptible to *S. bombi* infection, queens appeared to show a level of resistance to the castrating impacts of *S. bombi* that the parasite has in other bumblebee species, and were capable of laying eggs and, more rarely, founding colonies and producing males and gynes (**Figure 3.6**; **Figure 3.7**). Further, despite successful infection and maturation inside *B. hypnorum* queens (**Figure 3.3**), *S. bombi* appeared to be restricted in the completion of its life-cycle, with successful reproduction inside the host queen occurring very rarely, and larval stages never being passed in the host faeces or being found in the host hindgut (**Table 3.2**). The potential impacts of these interactions from both the host and parasite perspective are discussed below.

(a) Parasite prevalence in the UK *B. hypnorum* population

Parasite screening results from the present study confirm the previous findings of Jones & Brown (2014) that *B. hypnorum* shares a parasite prevalence and community structure similar to those of other native UK bumblebee species. It is therefore highly unlikely that the successful colonisation of the UK by *B. hypnorum* has been facilitated through escape from parasites, and hence the results of the present study show that the UK *B. hypnorum* population does not provide support for the enemy release hypothesis (Keane & Crawley, 2002; Torchin *et al.*, 2003). Specifically, the present study confirmed the presence of the four generalist bumblebee parasites *A. bombi*, *C. bombi*, *N. bombi*, and *S. bombi* in the *B. hypnorum* population at prevalences of 3.1%, 23.1%, 1.5%, and 16.5%, respectively (**Figure 3.4A**). Given the generalist nature of these parasite species (Schmid-Hempel, 2001), it is likely that parasite prevalence in the UK *B. hypnorum* population results from its sharing its parasite community with native *Bombus* species, with *B. hypnorum* acquiring parasites from native UK bumblebees and vice versa through the shared use of both foraging and hibernation sites (Alford, 1969a,b; Durrer & Schmid-Hempel, 1994). Nonetheless, parasite prevalence has seemingly done very little to hamper *B. hypnorum*'s rapid spread across the UK (**Figure 1.2**).

Comparisons between the present study and Jones & Brown (2014) revealed similar *C. bombi* prevalence across both studies (present study: 60/260; Jones & Brown (2014): 14/59), such that there was no significant difference in prevalence between the two studies (Fisher's exact test: $p = 1$). Further, Jones & Brown (2014) found *N. bombi* to be absent from *B. hypnorum*, whereas the present study confirmed its existence in the UK population, where it occurred in 4/260 queens (**Figure 3.4A**). However, such differences were non-significant (Fisher's exact test: $p = 1$) and given the rarity of *N. bombi* in the present study, it is likely that non-discovery in Jones & Brown (2014) was likely due to a smaller sample size rather than its actual absence at the time and location of sampling. However, both *A. bombi* and *S. bombi* occurred at significantly lower prevalences in the present study (present study: *A. bombi* = 8/260; *S. bombi* = 43/260; Jones & Brown (2014): *A. bombi* = 11/59; *S. bombi* = 17/59; Fisher's exact test: *A. bombi* $p < 0.001$, *S. bombi* $p = 0.04$). Such differences are likely to be due to the sampling of *B. hypnorum* queens being carried out in different years and locations, and demonstrate the fluctuating nature of parasite communities (e.g. Lockley *et al.*, 2020), underlining the importance of long-term parasite monitoring to gain greater insight into host-parasite interactions (Penczykowski *et al.*, 2016).

(b) *Sphaerularia* impacts on *B. hypnorum* queen fitness

In the present study, quantification of the fitness impacts associated with *S. bombi* infection demonstrate that, while *B. hypnorum* queens are vulnerable to *S. bombi* infection, they exhibit partial resistance to the castrating effects of the nematode, confirming previous anecdotal evidence of this phenomenon (Hasselrot, 1960; Röseler, 2002; Jones & Brown, 2014). Specifically, *S. bombi* queens were just as capable as uninfected queens of laying eggs, with 71.4%

of the infected queens from the 2019 cohort laying eggs and hence demonstrating ovarian activity (**Figure 3.6**). Further, infected queens were also capable of both adult offspring production and colony foundation, with 18.6% (8/43) of queens producing at least one adult offspring and 7% (3/43) of queens producing 5 or more adult workers, respectively (**Figure 3.6**). Further, of the 8 adult offspring-producing queens, 6 managed to produce adult males and a single queen managed to produce adult gynes (**Figure 3.7**). Hence, 13% (6/43) of the *S. bombi*-infected queens were capable of sexual production. Such patterns are in stark contrast to the castrating effect of *S. bombi* in other *Bombus* species, with *S. bombi* infection completely preventing egg-laying in *B. pratorum* (Rutrecht & Brown, 2008), and reducing ovarian activation and preventing offspring production in both *B. lucorum* and *B. terrestris* (Kelly, 2009). A caveat to the current study is that queens were reared under controlled conditions, with food provided *ad libitum*, potentially masking the fitness costs of *S. bombi* infection in nature. However, *S. bombi* castration of *B. lucorum*, *B. pratorum*, and *B. terrestris* were all observed under controlled, captive conditions (Rutrecht & Brown, 2008; Kelly, 2009), so the point still stands that *B. hypnorum* exhibits some resistance to the castrating effects of *S. bombi*. Hence, that a subset of *B. hypnorum* queens can still successfully produce sexual offspring despite infection with *S. bombi*, rather than being completely lost from the population (as in other bumblebee species), may therefore represent one factor underlying the ecological success and rapid range expansion of *B. hypnorum* in the UK (**Figure 1.2**).

While *B. hypnorum* queens may be partially resistant to the castrating effects of *S. bombi* infection, *S. bombi* infection still comes at a significant cost to queen fitness when compared to uninfected queens. Despite no difference in successful egg-laying between uninfected and *S. bombi*-infected queens, *S. bombi* infection led to queens being less successful in offspring production and colony foundation than uninfected queens (**Figure 3.6**). This finding suggests that while infected *B. hypnorum* queens can still develop their ovaries and lay eggs, infection with *S. bombi* compromises their ability to rear eggs to adulthood. This could potentially be due to *S. bombi*-related changes in host behaviour, which are well-documented in *S. bombi*-infected bumblebees (Poinar Jr & Van der Laan, 1972; Lundberg & Svensson, 1975) along with hornet (*Vespa simillima*) queens infected with the closely-related *Sphaerularia vespae* (Sayama *et al.*, 2013). In such cases, *Sphaerularia* infection causes queens (in nature) to search for potential overwintering sites in which to deposit juvenile nematodes instead of founding nests (Poinar Jr & Van der Laan, 1972; Lundberg & Svensson, 1975; Sayama *et al.*, 2013), leading to completion of the parasite life cycle (**Figure 3.1**).

Further fitness costs associated with *S. bombi* infection were also observed across offspring-producing queens, with infection leading to queens producing fewer workers, males, and gynes than uninfected queens (**Figure 3.7**; **Table 3.1**). Reductions in host reproductive success are commonly observed following parasite infection (Fitze *et al.*, 2004; Rutrecht & Brown, 2008; Lockley *et al.*, 2020), with such reductions attributable to host life-history trade-offs between reproduction and immune responses (Forbes, 1993; Sheldon & Verhulst, 1996). Finally, *S. bombi*-infection also led to a reduction in *B. hypnorum* queen longevity, with infected queens living a median of 8 days less than uninfected queens (**Figure 3.8**). Similar reductions in queen longevity were also found in *B. lucorum* and *B. terrestris* but were not present in *B. pascuorum* or *B. pratorum* (Kelly, 2009), suggesting that *S. bombi* may have species-specific impacts on host longevity. That *S. bombi* reduces the longevity of the host is somewhat surprising, given that *S. bombi* is reliant on the host queen to visit and deposit larval nematodes at hibernation sites (**Figure 3.1**) and hence reduced host longevity may ultimately reduce parasite reproductive success. One potential explanation for reduced host longevity may be due to infection intensity, with larger parasite loads leading to reduced queen longevity, as documented in both *B. lucorum* and *B. terrestris* (Kelly, 2009). Interestingly, no pattern between infection intensity and queen lifespan was found in the present study (**Figure 3.9**). However, this pattern might have been missed due to the small sample sizes of *B. hypnorum* queens with high infection intensities (i.e. only 6/43 queens displayed infection intensities of higher than 10), leading to reduced statistical power.

One further parasite-related mechanism that may play a role in the rapid ecological success of *B. hypnorum* is bivoltinism, whereby two colony cycles (and hence two generations) are achieved within a single year (Edwards & Jenner, 2005). Evidence for such behaviour in *B. hypnorum* comes from the observation of a second peak in worker abundance during late summer (Edwards & Jenner, 2005). Importantly, if *B. hypnorum* does exhibit bivoltinism, then the first generation of queens produced each year escape from *S. bombi* infection given that they begin nest foundation without having undergone hibernation, and hence are not exposed to the infective stages of *S. bombi*. Therefore, the first generation of queens would not suffer the negative fitness impacts imposed by *S. bombi*, potentially leading to increased ecological success. However, the existence of bivoltinism in *B. hypnorum* is yet to be quantitatively confirmed and hence the effects of bivoltinism on host-parasite interactions between *B. hypnorum* and *S. bombi* remain unclear.

(c) *Sphaerularia* reproductive success in *B. hypnorum*

Quantification of *S. bombi* prevalence through queen dissections in the present study demonstrates that *S. bombi* occurs at a frequency of 16.5% in the UK *B. hypnorum* population (**Figure 3.4**). This confirms that *S. bombi* females encounter *B. hypnorum* queens during hibernation (presumably due to the shared use of overwintering sites between *Bombus* species), are capable of infecting *B. hypnorum* queens, and can survive inside the haemocoel over the hibernation period. Further, all 198 *S. bombi* females found in the 43 infected queens had successfully everted and enlarged their uterus (**Figure 3.2E**; **Figure 3.3**; **Figure 3.5**), suggesting that, upon successful infection of the *B. hypnorum* host, females are also capable of undergoing the physiological changes associated with reproductive maturity

(Poinar Jr & Van der Laan, 1972). As in other *Bombus* species (e.g. Poinar Jr & Van der Laan, 1972; Rutrecht & Brown, 2008), multiple infection of hosts was commonplace (**Figure 3.3**; **Figure 3.5**), with 76.7% of infected *B. hypnorum* queens harbouring more than one mature *S. bombi* female and a mean infection intensity of 4.6 *S. bombi* females. Interestingly, measurement of each *S. bombi* female uncovered a negative relationship between infection intensity and uterus size (**Figure 3.5**), such that *S. bombi* females were larger when they encountered fewer conspecifics (i.e. lower infection intensity) once inside the queen host. Similar relationships are observed across tapeworm and nematode species that infect fish and mammalian hosts (Arneberg *et al.*, 1998; Poulin, 1999; Randhawa & Poulin, 2009), and can be explained by competition-dependent growth restrictions imposed on parasites due to the finite nature of host resources. To my knowledge, this is the first record of such a relationship in a nematode-insect system; future research could investigate the relationships between infection intensity and parasite reproductive success.

Assessment of the reproductive success of *S. bombi* inside *B. hypnorum* hosts demonstrated that *S. bombi* females very rarely successfully reproduce, with eggs and larvae found in the haemocoel of just 16.3% and 4.7% of infected queens, respectively (**Table 3.2**). These findings suggest that while *S. bombi* can infect and mature inside *B. hypnorum*, females only occasionally manage to release eggs into the host haemocoel, and these eggs rarely hatch into the L3 juvenile stage. Further, the lack of any L3 larvae in the hindgut of infected queens (**Table 3.2**) suggests that, even if *S. bombi* females produce eggs and these eggs hatch, the larvae cannot penetrate the host gut wall and hence are not transmitted in the host faeces. Such findings explain the lack of *S. bombi* larval transmission in queen faeces in the present study (**Table 3.2**) and in the study of Jones (2013) showing that infected *B. hypnorum* queens failed to pass any *S. bombi* larvae in the faeces up to 9 weeks after emergence from hibernation. *B. hypnorum* therefore appears to possess multiple physiological and immunological filters that impair *S. bombi* reproduction, and such mechanisms may explain *B. hypnorum*'s partial resistance to the castrating effect of *S. bombi*. Investigation into the transcriptomic changes associated with *S. bombi* infection in *B. hypnorum* (c.f. Colgan *et al.*, 2020) may provide a better understanding of the physiological and immunological responses that limit successful *S. bombi* reproduction.

Taken together, the findings of the present study suggest that infection of *B. hypnorum* represents a severe fitness cost to *S. bombi* in comparison to infection of queens of other *Bombus* species. Firstly, *S. bombi* fails to completely castrate infected queens (and hence manipulate them into larval deposition), and secondly, *S. bombi* only rarely manages to successfully reproduce once inside the queens. Hence, *S. bombi* is unlikely to be able to complete its life cycle (**Figure 3.1**) when infecting *B. hypnorum*, suggesting that *B. hypnorum* represents a partially dead-end host for *S. bombi*. One interesting effect of such a host-parasite relationship is that of 'parasite dilution', whereby a native parasite is acquired by a novel host that acts as a dead-end for parasite transmission, leading to native hosts experiencing reduced prevalence of the parasite in question (Telfer *et al.*, 2005; Kopp & Jokela, 2007). Given the castrating impacts of *S. bombi* in other native British bumblebee species (Rutrecht & Brown, 2008; Kelly, 2009), the arrival and dead-end host role of *B. hypnorum* could therefore be beneficial to other bumblebees. However, long-term monitoring of host-parasite interactions between *S. bombi* and multiple UK *Bombus* species (including *B. hypnorum*) would be required to test this idea.

(d) Conclusions

Overall, this study provides valuable insight into host-parasite interactions between the highly ecologically successful *B. hypnorum* and the generalist bumblebee nematode parasite, *Sphaerularia bombi*. Specifically, the findings support previous research (Jones & Brown, 2014) that the colonisation of the UK by *B. hypnorum* is not associated with escape from parasites encountered its native range. Further, novel findings of the study demonstrate that, in contrast to other *Bombus* species (Rutrecht & Brown, 2008; Kelly, 2009), *B. hypnorum* exhibits some resistance to the castrating effect of *S. bombi*, with such resistance potentially representing a factor behind *B. hypnorum*'s success in the UK. Finally, reduced reproductive success of *S. bombi* females upon infection of *B. hypnorum* suggest that *B. hypnorum* may represent a dead-end host for the nematode, and such interactions may benefit native bumblebees through parasite dilution. Ultimately then, these findings demonstrate the importance of quantifying host-parasite interactions across multiple species of bumblebees and other taxa, particularly pollinators, and their broad generalist parasite communities, especially in the face of ongoing global pollinator population declines.

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Chapter 4

Colony demography and queen lifetime reproductive success in the Tree Bumblebee (*Bombus hypnorum*)

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A Tree Bumblebee (*Bombus hypnorum*) queen (centre) surrounded by her individually marked daughter workers.

CHAPTER 4

Colony demography and queen lifetime reproductive success in the Tree Bumblebee (*Bombus hypnorum*)

4.1. Abstract

Our knowledge of colony demography and life-history in the eusocial Hymenoptera is limited. However, such knowledge is essential to understand relationships between life-history and reproductive and/or ecological success in this group, along with interactions between eusociality, ageing and longevity. Here, I present the first comprehensive study of colony demography in the Tree Bumblebee (*Bombus hypnorum*), allowing comparison with the native *B. terrestris* to highlight demographic features that may explain *B. hypnorum*'s rapid range-expansion across the UK, along with examination of the relationship between queen longevity and reproductive success in *B. hypnorum*. My findings highlight key similarities and differences in demographic traits between *B. hypnorum* and other annual eusocial species, with *B. hypnorum* exhibiting significantly higher levels of gyne (new queen) productivity than *B. terrestris*. Accordingly, high levels of gyne productivity may be linked to the range expansion and ecological success of *B. hypnorum* in the UK. Further, I show that queen lifetime reproductive success is positively associated with queen longevity, but is also influenced by other demographic traits, such as total worker production, colony lifespan, and latency to colony initiation. Such findings corroborate previous studies in both annual and perennial eusocial species, suggesting that positive relationships between longevity and reproductive success may be universal across the eusocial Hymenoptera. Taken together, these findings highlight the importance of a knowledge of colony demography and life-history in understanding the ecological success of a key group of pollinators and the evolution of life-history in eusocial organisms.

4.2. Introduction

Identifying the morphological, behavioural and life-history traits that influence an individual's reproductive success is essential to address fundamental questions in evolutionary biology (Howard, 1979; Clutton-Brock, 1988). Of particular interest is the life-history trade-off observed between longevity and reproduction in animals (Jones *et al.*, 2014), which stems from the limited resources an organism can allocate to either somatic maintenance or reproduction (Maklakov & Immler, 2016), and how this trade-off is influenced by sociality (Dammann & Burda, 2006; Bourke, 2007; Downing *et al.*, 2021; Korb & Heinze, 2021).

Eusocial insects (ants, bees, wasps, and termites with a queen and worker caste) exhibit an apparent reversal of the fecundity-longevity trade-off, whereby reproductive castes are vastly longer-lived than non-reproductives (Keller & Genoud, 1997; Heinze & Schrempf, 2008; Kreider *et al.*, 2021; Tasaki *et al.*, 2021), and fecundity and longevity are positively associated (Hartmann & Heinze, 2003; Heinze & Schrempf, 2012; Rueppell *et al.*, 2015; Blacher *et al.*, 2017; Schrempf *et al.*, 2017). However, few studies to date have linked queen longevity to realised reproductive success (in terms of total sexual output) in eusocial species (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015). This is more broadly reflective of the paucity of colony-level, life-history data across eusocial insects in general, despite the value of such data to the study of social insect evolution (Tschinkel, 1991; Cole, 2009). Such knowledge gaps are likely to be due to the difficulty in gathering lifetime demography data, given that eusocial colonies are often extremely long-lived, highly populous, hard to access (e.g. through the nest being underground), and may not thrive under captive conditions (Tschinkel, 1991; Cole, 2009). Therefore, colony demographic studies are most suited to annual eusocial species (such as bumblebees and paper wasps; Duchateau & Velthuis, 1988; Müller & Schmid-Hempel, 1992; Clapperton & Dymock, 1997) or perennial ants with small and relatively short-lived colonies (Heinze, 2017), with previous studies in such systems finding positive associations between queen longevity and reproductive success (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015). However, similar studies across additional species are required to confirm whether such relationships are the rule across eusocial insects.

Bumblebees (*Bombus* spp.) comprise approximately 250 species of obligately eusocial or socially parasitic species, distributed across the Americas, Eurasia and northern Africa (Williams *et al.*, 2008; Goulson, 2009). Bumblebees can be considered intermediately eusocial (Harrison *et al.*, 2015), sharing traits with both complex and simple eusocial societies (Bourke, 2011), such as pre-imaginal, size-based morphological queen-worker differentiation (Amsalem *et al.*, 2015), relatively small colony sizes, and high worker reproductive potential (Bourke, 1988; Zanette *et al.*, 2012). Across temperate zones, bumblebees exhibit annual colony cycles (**Figure 1.1**), with colonies being founded by single queens (monogyny) following their emergence from hibernation in the spring (Alford, 2011). Queens initially produce workers during the growth phase, before transitioning to the production of new queens (gynes) and males during the reproductive phase (**Figure 1.1**). The newly produced gynes and males then leave the colony to mate, after which the mated queens enter hibernation while the males and remaining workers eventually die, bringing the colony cycle to an end (**Figure 1.1**).

The Buff-Tailed Bumblebee (*B. terrestris*) represents the best-studied bumblebee to date, with numerous demographic studies having been conducted across multiple populations from its native Palaearctic range (e.g. Duchateau & Velthuis, 1988; Müller *et al.*, 1992; Beekman & Van Stratum, 1998; Duchateau *et al.*, 2004; Lopez-Vaamonde *et al.*, 2009), leading to a thorough understanding of colony demography in this species. Colonies initially follow a step-wise growth pattern as queens lay two separate broods of diploid, worker-destined eggs, before transitioning to continuous egg-laying, resulting in linear colony growth (Duchateau & Velthuis, 1988). Following this, colonies pass through two closely linked and key events, which mark the transition from investment in colony growth to investment in colony reproduction: these are the ‘switch point’ (when queens begin to lay haploid, male-destined eggs) and the onset of gyne production (Duchateau & Velthuis, 1988; Duchateau *et al.*, 2004), both of which appear endogenously controlled by the queen (Alaux *et al.*, 2005; Holland *et al.*, 2013). The switch point is characterised by a transition to 100% haploid egg production by the queen over a period of roughly ten days, and is closely followed by the ‘competition point’, whereby workers begin to compete with the queen over male production by laying their own haploid, male eggs and/or behaving aggressively towards the queen and other workers (Duchateau & Velthuis, 1988; Lopez-Vaamonde *et al.*, 2009). In some cases, conflict may escalate to the extent of workers killing the queen to gain control over male production (‘worker matricide’; Bourke, 1994), and may therefore influence queen longevity and reproductive success (Lopez-Vaamonde *et al.*, 2009; Almond *et al.*, 2019). Hence, while demography is well-studied in *B. terrestris* it remains to be seen whether such phenomena also occur in other annual eusocial Hymenoperan species, with comparisons of life-history strategies between species being essential to provide insight into their social evolution.

The Tree Bumblebee (*B. hypnorum*) is an ecologically successful, range-expanding bumblebee, with historical records suggesting a westward range-expansion across continental Europe during the 20th century (Wagner, 1937; Rasmont, 1989) before its arrival and spread across the UK and Iceland in 2001 and 2008, respectively (Goulson & Williams, 2001; Prŷs-Jones *et al.*, 2016). However, little is known with regards to the colony demography of *B. hypnorum*, and such knowledge may help understand its striking ecological success across new ranges. Hence, the aim of the present study was to provide the first comprehensive characterisation of colony demography for *B. hypnorum* and compare colony-level sexual productivity to that of *B. terrestris*, an abundant bumblebee that exhibits stable population trends across the UK and continental Europe (Casey *et al.*, 2015; Guzman *et al.*, 2021), to assess demographic differences that may represent a factor in the range-expansion success of *B. hypnorum*. Previous field-based observations of *B. hypnorum* nests have uncovered higher rates of successful gyne production (i.e. the proportion of colonies that successfully produce gynes) when compared to native bumblebees (Goulson *et al.*, 2018). Hence, I predicted that, relative to *B. terrestris*, *B. hypnorum* will exhibit higher rates of successful gyne production and higher levels of gyne productivity (i.e. the mean number of gynes produced per colony).

A further aim of this study was to establish the relationship between queen longevity and reproductive success in *B. hypnorum*. Eusocial societies, including those of bumblebees, often contain multiple parties with conflicting evolutionary interests over life-history strategies (queens, reproductive workers and non-reproductive workers; e.g. Ratnieks *et al.*, 2006). Current evidence suggests that *B. hypnorum* queens exhibit a large degree of control over sex allocation and male production (Paxton *et al.*, 2001; Brown *et al.*, 2003; **Chapter 5**). For instance, due to effective queen policing, workers contribute relatively little to a colony's sexual productivity, with 13.4% of adult males from the queenright, competition phase of the colony cycle being worker-produced (**Chapter 5**). These patterns of reproductive decision-making are also more broadly observed across other bumblebee species (e.g. Müller *et al.*, 1992; Alaux *et al.*, 2005; Holland *et al.*, 2013). Hence, the present study focusses solely on queen lifetime reproductive fitness. As in other similar studies (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015), I hypothesised that a positive relationship exists between queen-longevity and total reproductive success, but that this relationship is also influenced by other important demographic traits in bumblebees, such as colony size, colony longevity, and parasite infection status.

4.3. Methods

(a) Colony rearing and parasite screening

B. hypnorum colonies were reared from three cohorts of field-collected queens (n = 308) captured between 4 March and 8 April 2017, 7 March and

19 April 2018 and 25 February and 9 April 2019, respectively. The queens in the 2017 queen cohort (*n* = 45) were collected from West Wickham, Cambridgeshire (*n* = 2), Woolpit, Suffolk (*n* = 9) and various sites across Norwich, Norfolk (*n* = 34). The queens in the 2018 queen cohort (*n* = 107) were collected from Great Windsor Park, Surrey (*n* = 25), Chingford, Greater London (*n* = 7), and Norwich (*n* = 75). The queens in the 2019 queen cohort (*n* = 156) were collected from Norwich. Queens bearing pollen on their corbiculae were not collected, since such queens are likely to have already founded nests and so may not found nests under captive conditions (Tripodi & Strange, 2019). Previous research (Huml *et al.*, 2021) showed no genetic differentiation between UK *B. hypnorum* subpopulations. Hence, in the present study, all queens collected across all sites were pooled and considered representative of the UK *B. hypnorum* population.

Once collected, queens were transferred to custom-made acrylic queen-rearing boxes (dimensions: L14 × W8 × D5 cm; Signforce Ltd, Norwich, UK) and kept at constant conditions (mean ± range: 27°C ± 1°C; 60% ± 10% RH), with pollen (Sussex Wholefoods, Eastbourne, UK) and 50% Apiinvert sugar syrup (Südzucker AG, Mannheim, Germany) provided *ad libitum*. Queens/colonies were checked daily for offspring production (i.e. newly-eclosed adult bees) and, once colonies contained 10-20 workers, the queen, workers and brood were moved to wooden nest-boxes (dimensions: L30 × W20 × D17 cm), with pollen and sugar syrup again provided *ad libitum*.

Infection status for the queen cohorts reared in 2018 and 2019 was determined by screening queen faeces under an Olympus BX41 phase-contrast microscope (Olympus Corp., Tokyo, Japan) at 100-400× magnification (**Chapter 3**). Faeces were checked for four generalist bumblebee endoparasites known to infect *B. hypnorum* in the UK (Jones & Brown, 2014): (1) *Apicystis bombi*; (2) *Crithidia bombi*; (3) *Nosema bombi*; and (4) *Sphaerularia bombi* (**Figure 3.2**). Each 2018 queen was screened within 24 hours of collection and once again 7-10 days post-collection. Given that *S. bombi* larvae were never found in the faeces of the 2018 queens

(**Chapter 3**), the period between the first and second parasite screening was extended for the 2019 queens. Accordingly, each 2019 queen was screened within 24 hours of collection and once again 21 days post-collection. Infected queens/colonies were defined as those in which one or more of the four parasite species that were screened for was found, and uninfected queens/colonies were defined as those in which none of these four parasites was found. Cross-contamination between infected and uninfected queens/colonies was eliminated by keeping colony classes separate from one another and using separate sets of handling equipment for infected and uninfected colonies. Further potential contamination was reduced by the sterilisation of handling equipment (e.g. tongs) between the handling of colonies across both infected and uninfected groups. Besides this, infected and uninfected queens and colonies were treated identically.

S. bombi eggs and larvae did not appear to be shed in *B. hypnorum* queen faeces and were not found during faecal screening (**Chapter 3**). *S. bombi* infection was therefore verified by the post-death dissection of the queen's abdominal cavity under a Zeiss Discovery v12 stereomicroscope (Zeiss, Oberkochen, Germany) at 10-30× magnification. Given that *S. bombi* infection could not be determined by faecal screening, *S. bombi*-infected queens would have been considered as uninfected during the rearing experiment and reared alongside other genuinely uninfected queens. However, given the sterilisation of handling equipment in between handling of different colonies, and that bumblebee queens are only vulnerable to *S. bombi* infection during hibernation (Poinar Jr & Van der Laan, 1972), there would have been no chance of *S. bombi* cross-infection between queens.

(b) Colony demography and productivity

Queens that produced over 5 workers and did not exhibit first-brood male production (potentially indicative of diploid male production; **Chapter 2**; Brock et al., 2021) were defined as having successfully founded colonies and were included in the present study (*n* = 54). Colonies were checked for adult offspring production between 09:00 and 20:00 every day, with colony rearing experiments lasting from 17 April to 1 September 2017, 2 April to 25 July 2018 and 14 March to 1 September 2019. All adult workers were individually marked with numbered discs (EH Thorne Ltd, Market Rasen, UK) upon eclosion (emergence from pupa), with the day of first worker eclosion defined as day one of the colony cycle for each colony. All adult sexuals (males and gynes) were removed from the colony upon eclosion and frozen at -20°C, since, under natural conditions, sexuals disperse from the colony a few days after eclosion (Alford, 2011). The switch point, defined as the day on which the first haploid, male-destined egg was laid, was estimated for each colony as the day of first male eclosion minus 18 days, based on the egg-to-adult development time of 18 days for males and workers, and 23 days for gynes, reported in Röseler & Röseler (1974).

Colonies were monitored daily for any dead individuals (colony queen or workers), which, once their individual identities and date of death had been recorded, were removed from the colony and frozen at -20°C. Some dead workers (n = 574; 16.1%) were found under the brood mass at the end of the rearing experiment, and longevity data were not available in such cases. Worker longevity was defined as the time in days between eclosion and death. Queen longevity was defined as the time in days between first worker eclosion and her death. Finally, colony longevity was defined as the time in days between first worker eclosion and last offspring eclosion (i.e. when colonies were no longer actively producing any brood).

To determine the population-level per capita mean weights of workers, males and gynes, a total of 222 workers ($n_{colonies}$ = 28), 224 males ($n_{colonies}$ = 28) and 185 gynes ($n_{colonies}$ = 26) were randomly sampled from 28 of the 54 colonies (used in **Chapter 5**), dried at 60°C for four days, and weighed to the nearest 10th of a milligram (BDH Balance Model 100A, Milton Keynes, UK).

Colony-level sexual mass (biomass in grams) was calculated for each colony as the sum [total number of gynes produced × population-level mean per capita weight of a gyne] plus [total number males produced × population-level mean per capita weight of a male]. Colony-level sexual mass therefore includes all males produced under both queenright and queenless conditions, where 'queenright' means in the queen's presence. This term was then used to calculate reproductive investment, defined as colony-level sexual mass divided by the total offspring mass, given for each colony as: colony-level sexual mass plus [total number of workers produced × population-level mean per capita weight of a worker]. Finally, sex investment ratios, defined as the total gyne mass divided by the colony-level sexual mass, were calculated for each colony.

(c) Productivity comparisons between *B. hypnorum* and *B. terrestris*

To investigate potential factors of ecological success linked to the colony demography of *B. hypnorum*, I compared colony-level measures of productivity between the *B. hypnorum* colonies in the current study to those taken from 58 *B. terrestris* colonies reared in previous studies (Lopez-Vaamonde *et al.*, 2004; Raine *et al.*, 2006). The *B. terrestris* colonies reared in Lopez-Vaamonde *et al.* (2004) and Raine *et al.* (2006) were selected for comparison since they represent the only large-scale demography dataset of colonies reared from field-collected queens for any UK bumblebee species, with captive rearing techniques similar to those used in the present study. Importantly, *B. terrestris* exhibits stable population trends in the UK and continental Europe (Casey *et al.*, 2015; Guzman *et al.*, 2021) and so offers an informative contrast with respect to demographic features linked to the range expansion of *B. hypnorum*.

Firstly, I tested for differences in the proportion of colonies that successfully produced either males or gynes between the two species (i.e. are *B. hypnorum* colonies more likely to successfully produce males or gynes than *B. terrestris*?). I calculated proportions as the number of colonies that successfully produced males or gynes across the total number of colonies reared for each species. Secondly, I tested for differences in numeric per worker productivity for male, gyne and sexual production between the two species (i.e. are *B. hypnorum* workers more efficient at converting resources into males or gynes than *B. terrestris*?). Here, per worker productivity was calculated as the total number of males, gynes or sexuals produced by each colony divided by the total number of workers produced by each colony. Differences between *B. hypnorum* and *B. terrestris* were tested using univariate analyses (detailed below).

(d) Queen lifetime reproductive success

Queen lifetime reproductive success (biomass in grams) was defined, for each colony, as the sum: [total number of gynes produced × population-level mean per capita weight of a gyne] plus [total number of queen-derived males produced × population-level mean per capita weight of a male]. Queen-derived males were classified as all those that eclosed up to 18 days post-queen death, i.e. those males that would have developed from eggs laid under queenright conditions. In **Chapter 5**, I found that, under queenright conditions, workers gain an estimated 13.4% share of male parentage across those eggs laid after the competition point. However, given that the competition point coincides with the beginning of male eclosion (**Figure 5.1**), total worker contribution to male production under queenright conditions is likely smaller than 13.4%, since the queen would have already laid many male-destined eggs prior to the competition point. Hence, the presence of worker-produced males within the cohort of males eclosing under queenright conditions would have negligible effects on the analysis.

(e) Statistical analyses

All statistical analyses were carried out using R v4.0.1 (R Core Team, 2020), with all data visualised using the *ggplot2* R package (Wickham, 2009).

A Shapiro-Wilk test was used to determine whether switch-points followed a continuous or bimodal distribution (i.e. whether queens can be sorted into early- and late-switchers). Differences in the proportion of colonies producing males or gynes between *B. hypnorum* and *B. terrestris* (Lopez-Vaamonde *et al.*, 2004; Raine *et al.*, 2006) were tested using Chi-squared tests. Differences between the per worker sexual productivity of *B. hypnorum* and *B. terrestris* colonies were tested using Wilcoxon rank sum tests.

To determine the influence of multiple demographic features on queen lifetime reproductive success, queen lifetime reproductive success was fitted as the response variable in a generalised linear model with Gaussian distribution and a log link function. Predictor functions were selected based on logical potential predictors of reproductive success identified from previous studies in bumblebees (Duchateau & Velthuis, 1988; Müller & Schmid-Hempel, 1992a; Rutrecht & Brown, 2008; Lopez-Vaamonde *et al.*, 2009), with the following variables being included in the initial, maximal model: queen longevity, colony longevity, total number of workers produced, switch point timing, infection status (uninfected vs infected) and the latency to colony initiation (defined as the time between queen collection and first worker eclosion). The minimal adequate model was then selected from this maximal model using the *dredge()* function in the *MuMIn* R package (Barton, 2020), which searches through all possible predictor combinations within the model and reports values of the sample size-corrected Akaike information criterion (AIC_c; Hurvich & Tsai, 1993). The candidate model with the lowest AIC_c was then reported as the best model. The adjusted R^2 value was calculated using the *rsq* R package (Zhang, 2017, 2021). Finally, a general linear model was used to test the relationship between worker longevity and the relative worker eclosion date within the colony cycle (i.e. whether later-eclosing workers have higher longevity or vice-versa). Model assumptions were visually assessed using the *autoplot()* function from the *ggfortify* R package (Tang *et al.*, 2016).

All means are presented ± 1 standard deviation unless otherwise stated.

4.4. Results

(a) Parasite infection, colony demography, and productivity

Of the 308 field-collected queens, 54 (17.5%) successfully founded colonies. Founding success rate differed between years, with 1 of 45 (2.2%), 19 of 107

(17.8%) and 34 of 156 (21.8%) queens successfully founding colonies from the 2017, 2018 and 2019 cohorts, respectively.

Of the 54 successful queens, 36 (66.7%) were uninfected, 15 (27.8%) were infected with *C. bombi*, 1 (1.9%) was infected with *S. bombi*, and 1 (1.9%) was infected with both *C. bombi* and *S. bombi*. Since the 2017 cohort was not screened for parasites, infection status could not be determined for the one successful 2017 queen, but post-death dissection confirmed that this queen was free of *S. bombi* infection.

Across the 54 colonies, the mean latency to colony initiation was 24.8 ± 4.0 (range: 19-35) days. Mean colony longevity was 78.5 ± 29.6 days and mean queen longevity was 59.6 ± 23.4 days (**Figure 4.1C**). Colonies were therefore under queenright conditions for a mean of 76.3% ± 20.9% of the colony cycle.

Colonies produced a mean of 66.2 ± 52.2 workers in total (**Figure 4.1A-B**), with the worker production period lasting a mean of 46.0 ± 14.3 days (**Figure 4.1C**). Colony growth patterns exhibited two distinct steps of worker production before transitioning to the continuous production of workers (**Figure 4.1A**), with a mean colony growth rate of 1.4 ± 1.1 workers eclosing per day during the worker production period.

Males were successfully produced by 50 (92.6%) of the 54 colonies, with a mean of 125.1 ± 114.9 males produced per colony (**Figure 4.1A-B**). Of these, 110.1 ± 108.0 (88.0%) were produced under queenright conditions, and 15.0 ± 35.6 (12.0%) were produced under queenless conditions (i.e. were exclusively worker-produced). On average, male production began on day 33.9 ± 9.6 (range: 18-76) of the colony cycle and lasted a mean of 48.7 ± 28.6 days (**Figure 4.1C**), with a mean male production rate of 2.6 ± 1.9 males eclosing per day during the male production period. Colonies contained a mean of 44.3 ± 38 (range: 4-148) workers at the onset of male production (**Figure 4.1D**). Minimum colony size for male production was five workers.

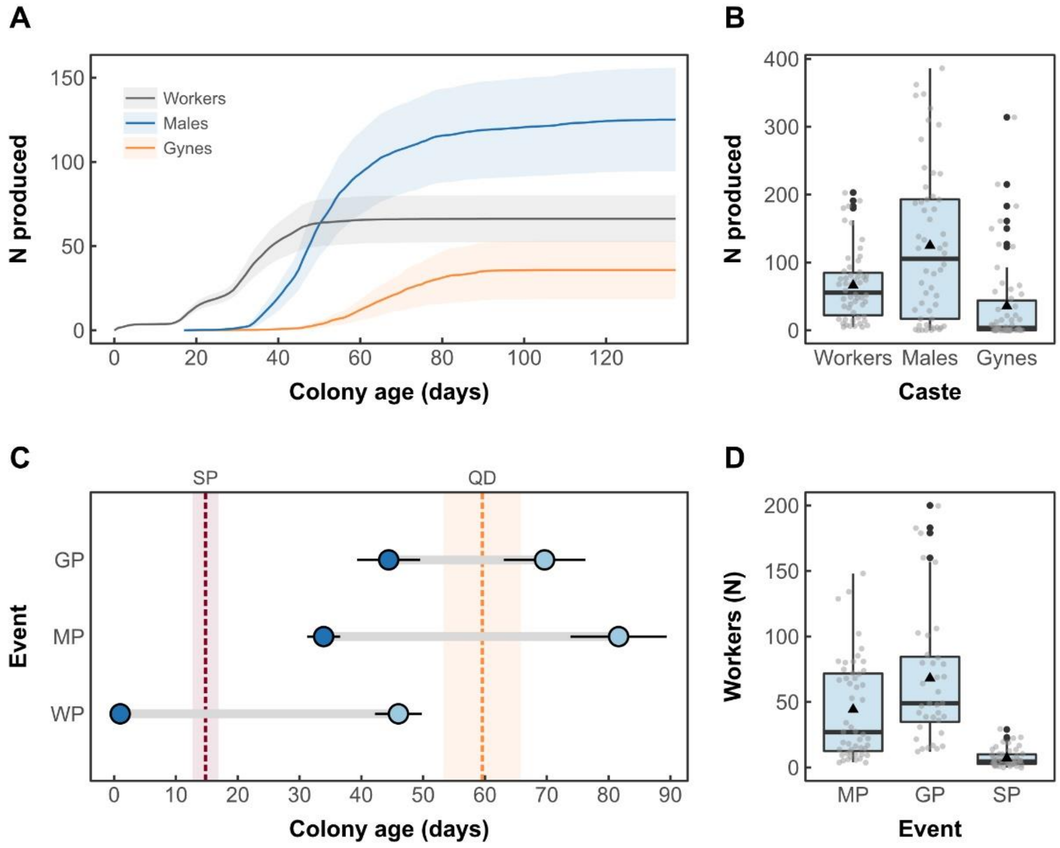

Figure 4.1. Colony demography and event timings for 54 Tree Bumblebee (*Bombus hypnorum*) colonies. **(A)** Mean timings and numbers (with shaded 95% confidence intervals) of workers (grey line), males (blue line) and gynes (orange line) produced by each colony. **(B)** Total numbers of workers, males and gynes produced by colonies. **(C)** Mean start (dark blue circles) and end points (light blue circles) with 95% confidence intervals (whiskers) of worker, male and gyne production ('WP', 'MP' and 'GP', respectively) throughout the colony cycle. Note there are no confidence intervals for the start of worker production since the day of first worker eclosion represents day 1 for every colony. Dashed burgundy line, mean day of switch point ('SP'), defined as the day when the first haploid egg was laid, for those queens that produced males (n = 48); dashed orange line, mean day of queen death ('QD'); shaded area around each dashed line, 95% confidence interval of event timing. **(D)** Number of workers within each colony at the onset of male production (first male eclosion), gyne production (first gyne eclosion) and the switch point. For boxplots **(B)** and **(D)**: thick horizontal bars, median; triangles, mean; boxes, interquartile range (IQR); filled black circles, outliers, defined as points more than 1.5 IQR above or below the upper or lower quartile; filled grey circles, 'jittered' raw data, with each point representing a single colony.

Gynes were successfully produced by 36 (66.7%) of the 54 colonies, with a mean of 35.8 ± 64.8 gynes produced per colony (**Figure 4.1A-B**). On average, gyne production began on day 44.4 ± 15.6 (range: 19-89) of the colony cycle and lasted a mean of 26.2 ± 22.2 days (**Figure 4.1C**), with a mean gyne production rate of 1.7 ± 1.5 gynes eclosing per day during the gyne production period. Colonies contained 68.2 ± 51.6 (range: 12-200) workers at the onset of gyne production (**Figure 4.1D**). Minimum colony size for gyne production was 14 workers.

Four (7.4%) of the 54 colonies failed to produce any sexuals, and mean colony size for these colonies was 44.0 ± 37.5 (range: 5-79) workers. Mean population-level per capita dry weights of workers, males and gynes were 36.3 ± 11.2 mg, 48.5 ± 13.4 mg and 135.5 ± 25.9 mg, respectively. Hence, per capita, gynes cost approximately 2.8× more than males. Overall, colonies committed a mean of 65.5% ± 29.5% of biomass produced towards sexual production, and, in the 50 colonies that successfully produced sexuals, the mean sex investment ratio was 0.24 ± 0.27, i.e. highly male-biased.

In two (4%) of the 50 colonies that successfully produced males, the first males eclosed more than 18 days after queen-death, so these males were likely to have developed from worker-laid eggs rather than from queen-laid eggs. Therefore, these two colonies were not used to calculate the switch point, with switch point calculation based on a total of 48 colonies. The mean switch point occurred on day 14.8 ± 7.4 of the colony cycle (**Figure 4.1C**, **Figure 4.2A**), and colonies contained a mean of 7.2 ± 6.9 (range: 0-29) workers at the switch point (**Figure 4.1D**).

There was no evidence to suggest that colonies were divided into early- and late-switchers, since the frequency distribution of the switch point did not significantly differ from a normal distribution (Shapiro-Wilk test: $W = 0.966$, $p = 0.18$; **Figure 4.2A**). The onset of the switch point was characterised by a sudden switch to haploid egg production by queens, with a mean of 64.2% ± 33.7% of eggs laid on the day of the switch point being haploid (**Figure 4.2B**). However, after the switch point, queens spent a long period of time (mean 29.8 ± 18.8 days) laying both haploid and diploid eggs (**Figure 4.2B**), with only 42 (87.5%) of the 48 queens transitioning to laying only haploid eggs before their death. Following the switch point, 76.9% ± 17.0% of all eggs laid by the queen were haploid (**Figure 4.2B**).

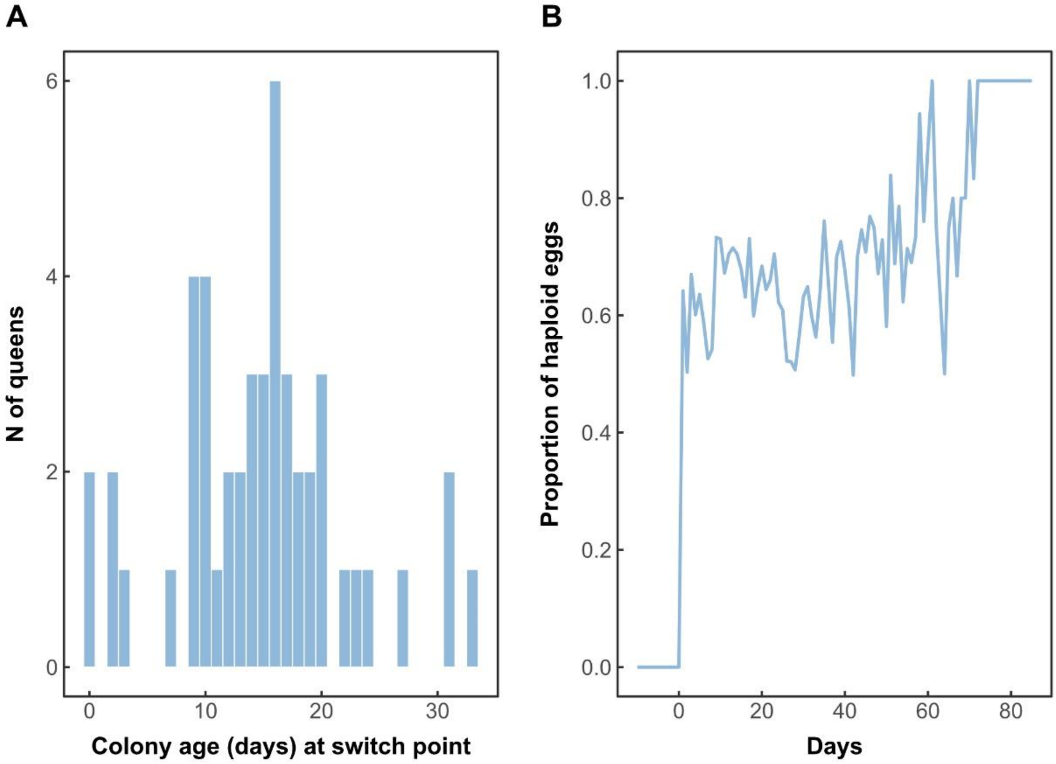

Figure 4.2. Timings and patterns of the switch point, defined as the day when the first haploid egg was laid, of 48 Tree Bumblebee (*Bombus hypnorum*) queens that successfully produced male offspring. **(A)** Distribution of switch point as a function of colony age (with day of first worker eclosion representing day 1). **(B)** Mean transition patterns and ploidy proportions of eggs laid as queens transitioned from laying diploid to haploid eggs. The proportion of haploid eggs (among queen-laid eggs) is inferred from the relative proportions of queen-produced males and gynes eclosing, back-calculated from male and gyne developmental time (Röseler & Röseler, 1974) to estimate the egg-laying date, as described in the text (mean ± SD diploid individuals for calculation per colony = 66.5 ± 90.3; mean ± SD haploid individuals for calculations per colony = 134.5 ± 109.7). The *x* axis is standardised to the switch point for each queen (i.e. day 1 represents the switch point for all queens).

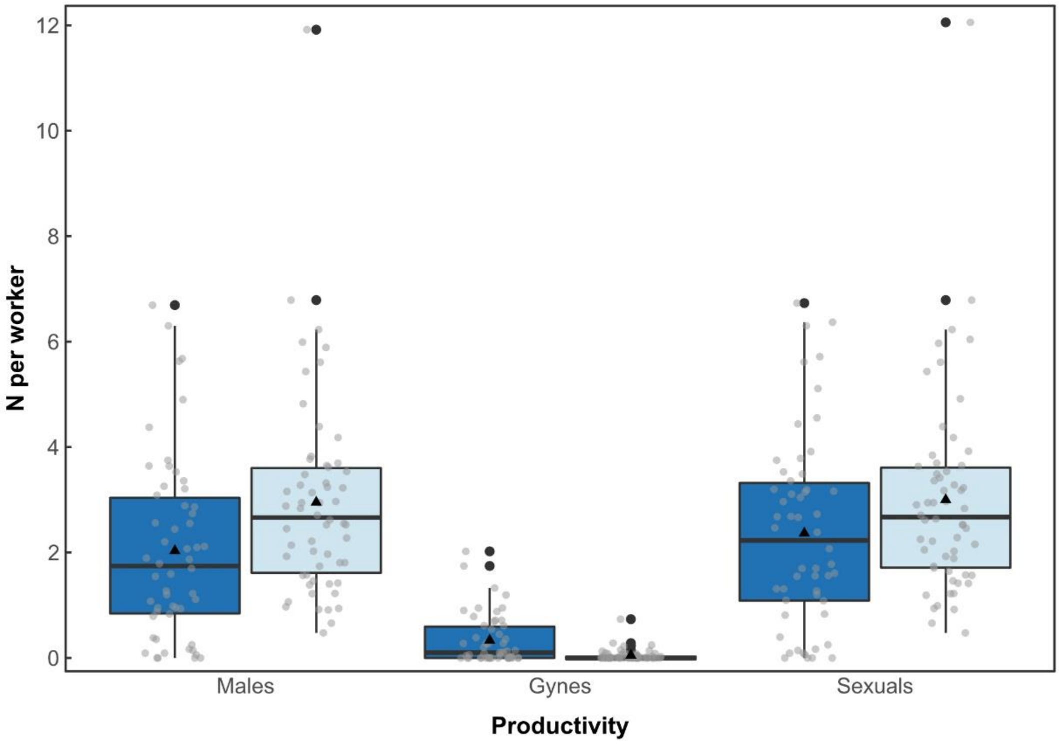

Figure 4.3. Per worker productivity differences between colonies of the Tree Bumblebee, *Bombus hypnorum* (navy; *n* = 54; data from present study), and the Buff-tailed Bumblebee, *Bombus terrestris* (light blue; *n* = 58; data from Lopez-Vaamonde *et al.*, 2004 and Raine *et al.*, 2006). Per worker productivity was calculated for each colony as the total number of males, gynes, or sexuals (males plus gynes) produced divided by the total number of workers produced. Thick horizontal bars, median; triangles, mean; boxes, interquartile range (IQR); filled black circles, outliers, defined as points more than 1.5 IQR above or below the upper or lower quartile; filled grey circles, 'jittered' raw data, with each point representing a single colony.

(b) Productivity comparisons between *B. hypnorum* and *B. terrestris*

There was no significant difference in the proportions of *B. hypnorum* and *B. terrestris* colonies that produced males (Chi-squared test: $\chi^2 = 2.56$, $p = 0.11$), with 92.6% (50/54) of the *B. hypnorum* colonies and 100% (58/58) of the *B. terrestris* colonies successfully producing males. Likewise, there was no significant difference in the proportions of colonies that produced gynes between the two species (Chi-squared test: $\chi^2 = 3.15$, $p = 0.08$), with 66.7% (36/54) of the *B. hypnorum* colonies and 48.3% (28/58) of the *B. terrestris* colonies successfully producing gynes.

B. hypnorum colonies produced significantly fewer males per worker than *B. terrestris* colonies (Wilcoxon rank sum test: W = 1063, $p = 0.003$), with *B. hypnorum* and *B. terrestris* colonies producing a mean of 2.0 ± 1.71 and 3.0 ± 1.9 males per worker, respectively (**Figure 4.3**). In contrast, *B. hypnorum* colonies produced significantly more gynes per worker than *B. terrestris* colonies (Wilcoxon rank sum test: W = 2159.5, $p < 0.001$), with *B. hypnorum* and *B. terrestris* colonies producing a mean of 0.3 ± 0.5 and 0.1 ± 0.1 gynes per worker, respectively (**Figure 4.3**). Overall, however, there was no difference in the number of sexuals produced per worker between the two species (Wilcoxon rank sum test: W = 1254, $p = 0.07$), with *B. hypnorum* and *B. terrestris* colonies producing a mean of 2.4 ± 1.8 and 3.0 ± 1.9 sexuals per worker, respectively (**Figure 4.3**).

(c) Queen lifetime reproductive success

Of the 48 queens that successfully produced sexuals, queen lifetime reproductive success was significantly positively associated with queen longevity ($t = 3.454$, $p = 0.001$; **Figure 4.4A**), total number of workers produced ($t = 10.564$, $p < 0.001$; **Figure 4.4B**), and colony longevity ($t = 3.672$, $p < 0.001$; **Figure 4.4C**), and negatively associated (although not significantly) with the latency to colony initiation ($t = -1.843$, $p = 0.072$; **Figure 4.4D**; **Table 4.1**). All four predictors were additive, such that queens who were longer-lived, produced a larger number of workers, headed longer-lived colonies, and were quicker to initiate their colonies achieved greater lifetime reproductive success (**Figure 4.4**), with the model explaining a high proportion of the variation in queenright total sexual biomass produced (Adjusted $R^2 = 0.854$; **Table 4.1**). Parasite infection and switch point timings were not included in the 'best' model as selected through AIC_c comparison (Hurvich & Tsai, 1993; Barton, 2020), suggesting that these predictors had negligible impacts on queen lifetime reproductive success.

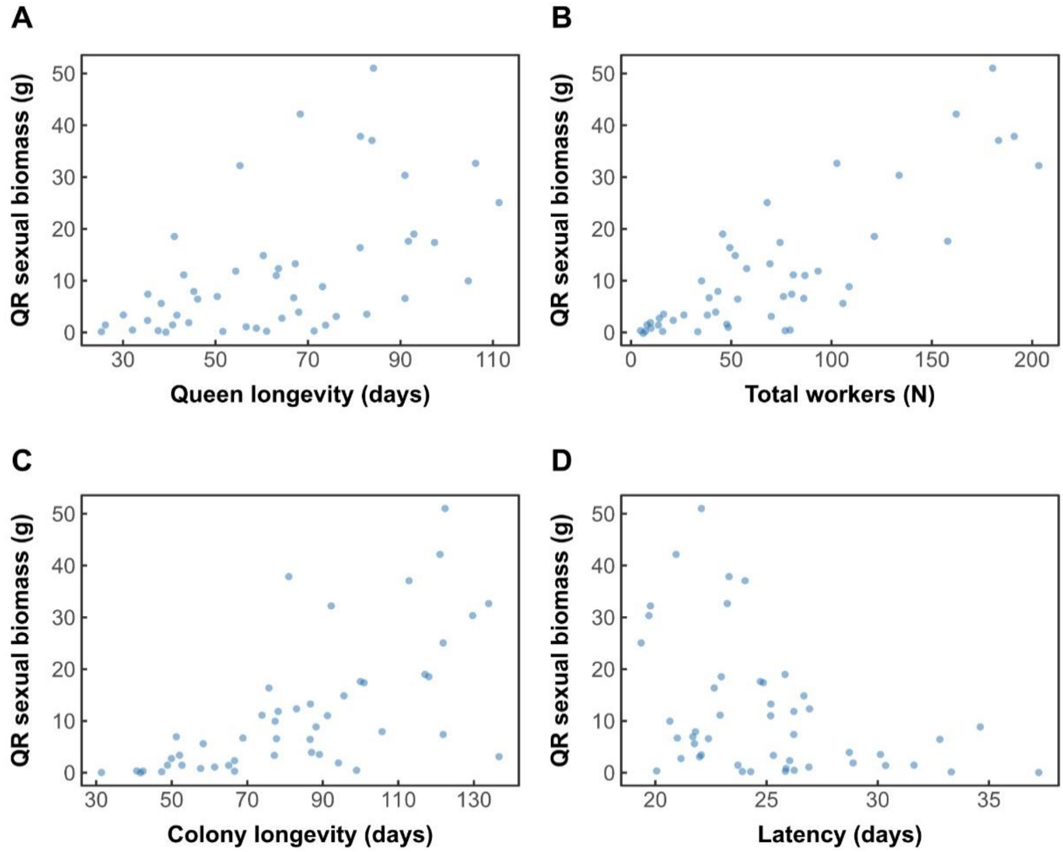

Figure 4.4. Queen lifetime reproductive success, measured as the total sexual biomass in grams produced under queenright ('QR') conditions, across 48 Tree Bumblebee (*Bombus hypnorum*) colonies as a function of: **(A)** queen longevity (defined as the time in days between first worker eclosion and queen death); **(B)** total number of workers produced by a colony; **(C)** colony longevity (defined as the time in days from first worker eclosion to day of last offspring produced); and **(D)** latency to colony initiation (defined as the time in days from queen collection to first worker eclosion). Each point represents a single queen/colony and, for illustrative purposes, each point is jittered to limit overlaps. The full model for queen lifetime reproductive success is summarised in **Table 4.1.**

(d) Worker longevity

Across all 54 colonies, worker longevities could be calculated for 3002 (83.9%) of all 3576 workers that were produced, comprising 84.7% ± 19.1% of the total workers per colony. The remaining 574 (16.1%) workers were not recovered until the end of the colony rearing experiment, and so longevity could not be calculated for these workers. Mean worker longevity was 40.5 ± 26.8 days.

There was no relationship between worker longevity and the relative worker eclosion date within the colony cycle ($F = 0.95$, $p = 0.33$; **Figure 4.5**), such that later-eclosing workers were not significantly longer-lived than early-eclosing workers.

Table 4.1. Model summary of *Bombus hypnorum* queen lifetime reproductive success (total sexual biomass in grams produced under queenright conditions) as a function of queen longevity, number of workers, colony longevity, and latency to colony initiation. Parameter estimates and hypothesis tests of all effects included in the 'best' generalised linear model selected using AIC_c (Hurvich & Tsai, 1993; Barton, 2020). Term, name of specified fixed predictor; Estimate, parameter estimate for each predictor; SE, standard error of estimate; *t*, t-test statistic; *p*, p-value, with a value of <0.05 indicating that the null hypothesis of the predictor effect being zero can be rejected.

Term	Estimate	SE	*t*	*p*
Intercept	0.878	0.640	1.371	0.178
Queen longevity	0.001	0.003	3.454	0.001
Total workers	0.001	0.001	10.564	<0.001
Colony longevity	0.001	0.003	3.672	<0.001
Latency	-0.038	0.021	-1.843	0.072
				Adjusted R^2 = 0.854

Queen longevity = the time in days between first worker eclosion and queen death.

Total workers = the total number of workers produced by each colony.

Colony longevity = the time in days between the eclosion of the first worker and the eclosion of the last sexual offspring (male or gyne) for each colony.

Latency = the time in days from queen collection to first worker eclosion.

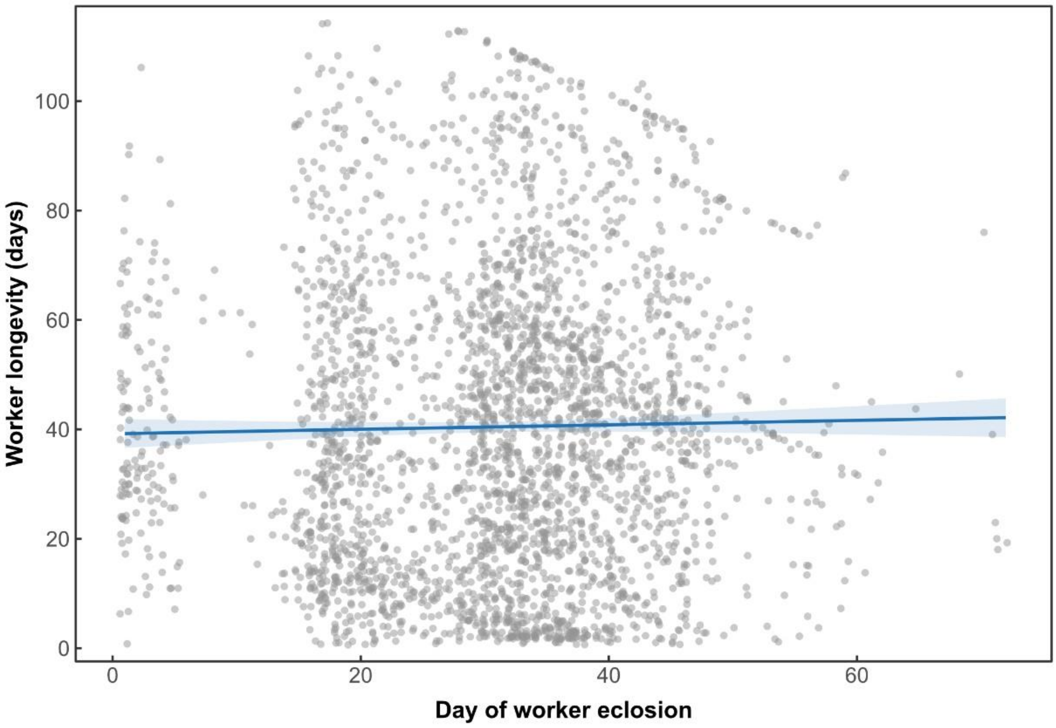

Figure 4.5. Worker longevity (defined as the time in days between eclosion and death) as a function of the relative day of worker eclosion in the colony cycle across 54 Tree Bumblebee (*Bombus hypnorum*) colonies. Navy line with shaded area, linear model (with 95% confidence interval) for worker longevity by day of worker eclosion ($F = 0.95$, $t = 0.98$, $p = 0.33$, $R^2 < 0.01$). Each grey point represents a single worker ($n = 3002$) and, for illustrative purposes, each point is jittered to limit overlaps.

4.5. Discussion

This study presents the first comprehensive characterisation of colony demography in the rapidly range-expanding and highly ecologically successful bumblebee pollinator, the Tree Bumblebee (*Bombus hypnorum*). In doing so, I was able to compare colony demography to that of *B. terrestris*, allowing the assessment of demographic features that potentially explain *B. hypnorum*'s range expansion, and investigate relationships between queen longevity and reproductive success. Results revealed several similarities and differences in demographic traits as compared with other bumblebee species and other annual eusocial Hymenopteran species (e.g. Greene, 1984; Duchateau & Velthuis, 1988; Müller & Schmid-Hempel, 1992a; Clapperton & Dymock, 1997), including, as predicted (see **Introduction**), higher levels of gyne-productivity when compared to *B. terrestris* (**Figure 4.3**). Further, as in previous studies, my findings confirmed the existence of a positive relationship between queen longevity and reproductive success in *B. hypnorum* (**Figure 4.4A**). The implications of these findings are discussed below.

(a) Colony demography and productivity

Colony growth and reproduction patterns observed in *B. hypnorum* (**Figure 4.1A**) are very similar to those reported from empirical studies in *B. terrestris* (Duchateau & Velthuis, 1988; Lopez-Vaamonde *et al.*, 2009) and largely conform to assumptions of theoretical models of optimal sexual production timings for bumblebees (Beekman *et al.*, 1998a). Specifically, *B. hypnorum* colonies show a stepwise growth pattern of worker production (corresponding to the two initial broods of worker-destined eggs laid by bumblebee queens; Duchateau & Velthuis, 1988; Beekman *et al.*, 1998a) before transitioning into the continuous production of workers, males and gynes (**Figure 4.1A**). Previous models of optimal sexual production strategies in annual eusocial societies predicted initial growth investment followed by a sharp switch to reproductive investment (the 'bang-bang' strategy; Macevicz & Oster, 1976; Bulmer, 1983b). However, such a pattern does not appear to be the case in *B. hypnorum* colonies, which are instead characterised by an initial period of exclusive worker production, followed by a period of mixed worker and sexual production, and a final period of exclusive sexual production (**Figure 4.1C**). Such patterns are also observed in *B. terrestris* and multiple annual eusocial wasps, suggesting ‘graded’ control of sexual production, with overlapping investment in both growth and reproduction in annual eusocial societies (Greene, 1984; Beekman *et al.*, 1998a; Johnson *et al.*, 2009; Poitrineau *et al.*, 2009), rather than explosive investment in sexual production at the end of the colony cycle (Macevicz & Oster, 1976; Bulmer, 1983b).

Typical of most other bumblebee species (with the exception of the socially parasitic *Psithyrus* subgenus; Bourke, 1997; Beekman & Van Stratum, 1998), *B. hypnorum* sex investment ratio was highly male-biased in the present study. Male-biased sex ratios in bumblebees represent an exception to sex ratios predicted by Trivers & Hare (1976) under both queen- and worker-control (0.5 and 0.75, respectively; **Figure 5.1**). However, male-biased sex ratios in bumblebees may be associated with protandry (the production of males before females), given patterns of bumblebee reproduction match the assumptions that lead to selection for protandry (Bulmer, 1983a,b; Bourke, 1997). Indeed, protandry was observed in the present *B. hypnorum* colonies (**Figure 4.1A**), and given the aggregation behaviour displayed at nest entrances by *B. hypnorum* males (Goulson *et al.*, 2018; Brock, *personal observation*), early-emerging males may gain greater mating opportunities than later-emerging males, leading to selection for protandry (Bulmer, 1983a,b; Bourke, 1997).

Switch-point timings and patterns in *B. hypnorum* showed similarities and differences compared with those in *B. terrestris* (Duchateau & Velthuis, 1988; Duchateau *et al.*, 2004). As in queens of *B. terrestris*, *B. hypnorum* queens in the present study demonstrated a clearly defined switch point, whereby, on the day of the switch point, haploid eggs accounted for 64% of all eggs laid (**Figure 4.2B**). However, unlike in *B. terrestris terrestris* populations (Duchateau *et al.*, 2004), there was no evidence for bimodal separation of the switch point in *B. hypnorum* (**Figure 4.2A**), and instead a normal distribution of switch points was found as in *B. terrestris audax* populations (Lopez-Vaamonde *et al.*, 2009). In addition, *B. hypnorum* queens did not switch to producing exclusively haploid eggs over a relatively short period, instead producing both haploid and diploid eggs over an extended timeframe (**Figure 4.2B**). This finding is reflected in the overlap of male production with both worker and gyne production schedules (**Figure 4.1C**). Interestingly, such patterns may influence kin-selected queen-worker conflict over male production in two ways. Firstly, in the absence of a sharper switch point, workers may avoid eating queen-laid eggs during the competition phase of the colony cycle, since they may risk eating valuable diploid, gyne-destined eggs (**Figure 5.1**), and this is consistent with the findings of **Chapter 5** that workers very rarely ate queen-laid eggs (**Figure 5.6**). Secondly, workers may be less inclined to commit matricide following the switch point (Bourke, 1994), since they risk killing a queen who is still producing female offspring (**Figure 5.1**). Accordingly, limited risk of worker matricide in *B. hypnorum* may lead to increased queen longevity and an associated increase in queen lifetime reproductive success (**Figure 4.4A**).

Successful colony foundation rates from field-collected queens were low in the present study (17.5% success rate overall), equivalent to success rates in other bumblebee species (e.g. Duchateau & Velthuis, 1988; Müller & Schmid-Hempel, 1992b). However, successful foundation rates increased over the three years this study took place, with success rates rising from 2.2% in 2017 to 21.8% in 2019. Such increases were likely to have stemmed from improvements in the husbandry of the colonies as the study progressed, combined with the use of parasite screening from 2018 onwards (**Chapter 3**). Further, even in widely-reared *Bombus* species, there is typically a relatively low upper limit to the percentage of wild-caught queens that successfully rear colonies in captivity (e.g. 26% in *B. terrestris* in Lopez-Vaamonde *et al.*, 2004). Interestingly, despite experiencing identical rearing conditions, *B. hypnorum* colonies exhibited striking variation in the total number of workers, males and gynes produced (**Figure 4.1B**), and similar variation has been shown in multiple other bumblebee species reared from field-collected queens (Müller & Schmid-Hempel, 1992a; Cnaani *et al.*, 2002; Duchateau *et al.*, 2004; Zhang *et al.*, 2018; Liang *et al.*, 2020). Such variation in productivity may therefore be the rule within bumblebees, and a better understanding of the life-history traits that influence it remains important.

Comparisons between the colony-level sexual productivity of *B. hypnorum* and *B. terrestris* in the present study found no differences in overall per worker sexual productivity between the two species (**Figure 4.3**). However, *B. hypnorum* appeared to invest more heavily in gyne production than *B. terrestris*, with 66.7% of colonies successfully producing gynes (versus 48.3% in *B. terrestris*) and *B. hypnorum* colonies exhibiting significantly higher levels of per worker gyne-productivity than *B. terrestris* (**Figure 4.3**). High levels of gyne productivity by *B. hypnorum* have been reported previously. For instance, 80% of colonies from a continental European *B. hypnorum* population produced gynes, with a mean of 58 gynes per colony (Brown *et al.*, 2003), similar to the mean of 36 gynes produced per colony in the present study (**Figure 4.1B**). Further, nest monitoring of seven British bumblebees (*B. hortorum*, *B. hypnorum*, *B. lapidarius*, *B. lucorum*, *B. terrestris*, *B. pascuorum* and *B. pratorum*) found that *B. hypnorum* had the highest gyne-rearing success rate, with 96% of 196 wild nests successfully rearing gynes (Goulson *et al.*, 2018). In comparison, laboratory-reared *B. terrestris* from UK populations produced a mean of 4-5 gynes per colony (Lopez-Vaamonde *et al.*, 2004, 2009; Raine *et al.*, 2006), *B. lucorum* from a Swiss population produced a mean of 3 gynes per colony (Müller & Schmid-Hempel, 1992b), and *B. impatiens* from a US population produced a mean of 9 gynes per colony (Cnaani *et al.*, 2002).

Given that queens represent the dispersing and founding propagule in bumblebees (and the majority of eusocial insects; Brown & Bonhoeffer, 2003; Cronin *et al.*, 2013), the high investment in gyne production by *B. hypnorum* may help explain its rapid range-expansion and ecological success in the UK (**Figure 1.2**). Indeed, the range-expanding nature of *B. hypnorum* may select for higher levels of gyne productivity, with those colonies that produce more gynes more likely to occupy previously unoccupied areas, and hence benefiting from reduced competition and higher fitness. However, the direction of this relationship, i.e. whether high gyne productivity leads to range-expansion or vice versa, remains unknown. Further, facultative bivoltinism (i.e. two colony cycles per year) has previously been suggested to occur in the UK *B. hypnorum* population (Edwards & Jenner, 2005), and may influence higher levels of gyne productivity in *B. hypnorum*. Here, the first-generation of queens may be selected to produce more gynes, given that this generation of queens can found colonies without overwintering, and hence would not suffer hibernation-associated mortality (e.g. Beekman *et al.*, 1998b; Straub *et al.*, 2015), further boosting the range-expansion of each lineage. Alternatively, apparent inter-specific differences in gyne investment may arise from differences in rearing techniques between studies (Velthuis & van Doorn, 2006; Owen, 2016), or the simple fact that *B. hypnorum* may adapt to captive conditions more readily than other bumblebee species (although given the relatively low rates of rearing success in the present study, this seems unlikely). Hence, rearing of multiple British bumblebee species under identical laboratory conditions such as those in Asada & Ono (2000), Zhang *et al.* (2018) and Liang *et al.* (2020) would be required to formally test this idea.

(b) Queen lifetime reproductive success

Demographic data collection across 54 colonies of *B. hypnorum* revealed positive associations between the longevity of a queen and her lifetime reproductive success, with longer-lived queens experiencing greater lifetime reproductive success (**Figure 4.4A**). Positive relationships between queen longevity and reproductive success have also been observed in the Buff-tailed Bumblebee (*B. terrestris*; Lopez-Vaamonde *et al.*, 2009) and two *Cardiocondyla* ant species (Heinze *et al.*, 2013; Kramer *et al.*, 2015), suggesting that this pattern exists across both annual and perennial eusocial insects. Such findings corroborate the well-reported positive association between reproduction and ageing in social insects (e.g. Keller & Genoud, 1997; Rueppell *et al.*, 2015; Kreider *et al.*, 2021), with social insect reproductives appearing to lack the fecundity-longevity trade-offs (i.e. negative association) shown in solitary insect species (e.g. Maynard Smith, 1958; Flatt, 2011), and animals more generally (Jones *et al.*, 2014).

Positive relationships between queen longevity and lifetime reproductive success in eusocial insects may stem from parental care traits exhibited by their solitary ancestors (Kelstrup *et al.*, 2018; Field *et al.*, 2020). Specifically, when parental care behaviour is present in solitary insects, increased female longevity should be selected for, since mothers can provide extended care for offspring and, in doing so, increase their own reproductive output and the fitness of their offspring (Field & Brace, 2004; Bourke, 2007; Field *et al.*, 2020). Indeed, such patterns are observed in the solitary bee *Osmia cornuta*, with longer-lived females able to provision larger numbers of offspring and benefit from increased reproductive success (Bosch & Vicens, 2006). Given parental care represents a necessary pre-adaptation for eusociality (Hunt, 1999; Field, 2005; Kelstrup *et al.*, 2018; Field *et al.*, 2020), it is likely that such positive relationships between longevity and reproductive success persisted in the transition to eusociality. In social insects, this relationship is further bolstered by the presence of workers, which assist in queen reproduction while simultaneously shielding the queen from extrinsic mortality (Keller & Genoud, 1997; Heinze & Schrempf, 2008). However, the testing of such an idea requires more life-history data across Hymenoptera at differing levels of social organisation.

In addition to queen longevity, queen lifetime reproductive success was also positively associated with colony size (i.e. the total number of workers produced; **Figure 4.4B**) and colony longevity (**Figure 4.4C**), and negatively associated with the latency to colony initiation (**Figure 4.4D**; **Table 4.1**). Positive relationships between worker production and queen reproductive success were also found in *B. terrestris* and two *Cardiocondyla* species (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015), while more broadly, larger colony sizes are associated with increased colony-level productivity in the eusocial Hymenoptera (Michener, 1964; Müller & Schmid-Hempel, 1992a). Such patterns are expected given that workers are responsible for the rearing of sexuals, with more workers leading to the successful rearing of more sexuals (although this pattern is non-linear, and ultimately limited by the rate at which queens lay sexual-destined eggs; Michener, 1964; Foster, 2004). Further, positive relationships found between colony longevity and queen reproductive success in *B. hypnorum* (**Figure 4.4C**) were also expected, given that the longer a colony survives for the more time it has to invest in sexual production (Lopez-Vaamonde *et al.*, 2009). Comparable negative associations between colony productivity and the latency to colony initiation also exist in *B. lucorum* (Müller & Schmid-Hempel, 1992a) and *B. ignitus* (Yoon *et al.*, 2004). As such, the relationship between increased queen reproductive success and latency to colony foundation may be explained by intrinsic differences in queen quality, with high-quality queens emerging from hibernation in better condition, leading to a smaller latency to colony foundation and increased reproductive success. However, the impacts of hibernation duration, queen condition, and subsequent reproductive success in bumblebees are understudied to date (Müller & Schmid-Hempel, 1992a; Beekman *et al.*, 1998b; Beekman & Van Stratum, 2000).

(c) Worker longevity

Finally, no significant relationship was found between longevity and relative eclosion date in *B. hypnorum* workers (**Figure 4.5**). This is in contrast to studies of other bumblebee species (Goldblatt & Fell, 1987; Holland & Bourke, 2015) that found negative relationships between worker longevity and relative time of eclosion (i.e. workers eclosing later in the colony cycle were shorter-lived). Such a contrast is potentially explained by the fact that workers in the present study were confined to their nest-boxes, whereas workers in the study of Goldblatt & Fell (1987) were free-foraging. Accordingly, later-eclosing workers from Goldblatt & Fell (1987) may have been more likely to become foragers and experienced higher extrinsic mortality rates as a result. Differences between the present study and Holland & Bourke (2015), in both of which workers were kept under captive conditions, may be due to species-specific worker longevity patterns, with an intrinsic negative relationship between worker-longevity and relative eclosion time present in *B. terrestris*, but not in *B. hypnorum*.

(d) Conclusions

In summary, this study shows positive relationships between queen longevity and queen lifetime reproductive success in the annual eusocial Tree Bumblebee (*B. hypnorum*). These findings match similar patterns across other annual and perennial eusocial Hymenoptera (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015), and suggest that such relationships may be universal across the eusocial insects, with longer-lived queens able to invest more time and resources into reproduction. Further relationships exist between queen reproductive success and worker production, colony longevity and the time to colony initiation in *B. hypnorum*, demonstrating that multiple colony life-history traits can influence queen fitness in social insects. Further, characterisation of *B. hypnorum* colony demography in the present study highlights relatively high investment in gyne production, which may help in explaining its rapid range-expansion across the UK, and allows the comparison of colony-level traits across annual eusocial species, with such data being essential for increasing understanding the evolution of sociality in such species.

4.6. References

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Chapter 5

Queen policing limits worker reproduction in the Tree Bumblebee (*Bombus hypnorum*)

A Tree Bumblebee (*Bombus hypnorum*) queen (left)

eats an egg laid by one of her daughter workers.

CHAPTER 5

Queen policing limits worker reproduction in the Tree Bumblebee (*Bombus hypnorum*)

5.1. Abstract

Conflict over male production is widespread in colonies of eusocial Hymenoptera since workers can lay haploid, male-destined eggs to which they are more related than their brothers (queen-produced males). Such conflict represents a dissolutive force during social evolution, and as such, ‘policing’ mechanisms are expected to evolve to resolve male production conflict. Here, I present the first behavioural analysis of male production conflict and its outcomes in a facultatively polyandrous annual eusocial Hymenopteran, the Tree Bumblebee (*Bombus hypnorum*). I found two mechanisms through which worker reproduction was limited by the actions of other individuals (policing): destruction of worker-laid eggs and interruption of egg-laying workers during oviposition through aggression. Surprisingly, policing by workers was rare across colonies, with policing being carried out almost exclusively by the queen. Collectively, workers were responsible for 64% of all eggs laid in the later part of the colony cycle. However, queen policing led to only 23.4% of worker-laid eggs remaining two hours after being laid. Accordingly, queen policing limited worker reproduction, with worker-produced adult males being detected (by parentage analysis using microsatellite markers) in 52% of colonies and comprising 13.4% of all adult males produced under queenright (i.e. in the presence of the queen), post-competition point conditions. Queen policing therefore acts to effectively suppress conflict in colonies of *B. hypnorum*, with these results demonstrating the diversity of conflict resolution strategies found across eusocial insect societies.

5.2. Introduction

Eusociality is an advanced form of social organisation in which colony members are divided into reproductive and non-reproductive castes, belong to two or more overlapping generations, and exhibit co-operative care of the young (Crespi & Yanega, 1995; Boomsma & Gawne, 2018). Reproductive success in eusocial societies is based on mutual dependence between castes, with such dependence being characteristic of a major evolutionary transition (Szathmáry & Smith, 1995; Bourke, 2011a; West *et al.*, 2015), as the non-reproductive individuals forgo reproduction to carry out tasks essential for colony productivity. Eusociality therefore represents an extreme form of altruism that, despite ongoing debate on the power and limits of inclusive fitness theory (e.g. Wilson & Hölldobler, 2005; Foster *et al.*, 2006; Nowak *et al.*, 2010; Bourke, 2011b, 2019; Allen *et al.*, 2013; Birch & Okasha, 2015; Kay *et al.*, 2020), is easily and elegantly explained by kin selection (see **Chapter 1**), whereby altruists increase their inclusive fitness by assisting the reproductive efforts of related individuals (Haldane, 1955; Hamilton, 1963, 1964a,b; Maynard Smith, 1964; Queller, 1992; Grafen, 2006).

The high levels of co-operation observed within eusocial societies have led numerous authors to describe them as ‘superorganisms’, with reproductive and non-reproductive castes analogous to the germ-line and soma of multicellular organisms (e.g. Wheeler, 1911; Seeley, 1989; Hölldobler and Wilson, 2009; Boomsma and Gawne, 2018). However, since such societies are often familial rather than clonal, fitness optima between colony members do not always align, and the potential for evolutionary conflict of interest (as predicted by kin selection theory) between individuals exists (Hamilton, 1964b; Trivers & Hare, 1976; Queller & Strassmann, 1998). Whether such conflicts are realised (become actual conflict) and how they are resolved is therefore a fundamental aspect of the evolution of eusociality, with kinship representing a major factor underlying conflict outcomes (Queller & Strassmann, 1998). To this end, due to their haplodiploid sex determination and diversity in kin structure, the eusocial Hymenoptera (all ants, bees and wasps with a worker caste) represent a highly informative model with which to test inclusive fitness-based predictions as to when co-operation and conflict occur (Trivers & Hare, 1976; Ratnieks & Reeve, 1992; Queller & Strassmann, 1998; Foster & Ratnieks, 2001a; Bourke, 2005; Oi *et al.*, 2020b).

Under haplodiploidy, females (queens and workers) develop from fertilised, diploid eggs, whereas males develop from unfertilised, haploid eggs (Cook & Crozier, 1995). Hence, while workers across the majority of eusocial Hymenoptera have lost the ability to mate and produce diploid, female offspring, they often retain functional ovaries and are therefore capable of laying haploid, male-destined eggs (Bourke, 1988). In fact, complete worker sterility is present in only a few ant and stingless bee genera (Fletcher & Ross, 1985; Boleli *et al.*, 1999; Dijkstra *et al.*, 2005; Bueno *et al.*, 2020), and consequently, potential conflict over male production is almost universal across the eusocial Hymenoptera (Bourke, 1988; Hammond & Keller, 2004).

Kin-selected conflict is predicted to occur over male production since, due to haplodiploidy, each female is more closely related to her own sons than she is to the sons of any other colony member (Hamilton, 1964b; Trivers & Hare, 1976). Specifically, in monogynous, monandrous colonies (colonies headed by one, singly-mated queen), the queen is more related to her own sons (r = 0.5) than to her workers' sons (r = 0.25), while workers are more related to their own sons (r = 0.5) or their fellow workers' sons (r = 0.375), than to their brothers (r = 0.25; **Figure 5.1**). Accordingly, the queen is selected to prevent her workers from successfully producing males, while workers are selected to bypass queen inhibition and replace queen-laid male eggs with their own eggs (Trivers & Hare, 1976; Ratnieks *et al.*, 2006). In extreme cases, conflict may lead to workers killing the queen to gain control over male production (Bourke, 1994; Loope, 2015; Giehr & Heinze, 2021). Further, the presence of reproductive workers within a colony may reduce colony productivity in a 'tragedy of the commons' scenario (Cole, 1986; Ratnieks, 1988; Hartmann *et al.*, 2003), whereby selfish individuals over-exploit a public good and eventually cause it to become exhausted (Hardin, 1968; Rankin *et al.*, 2007).

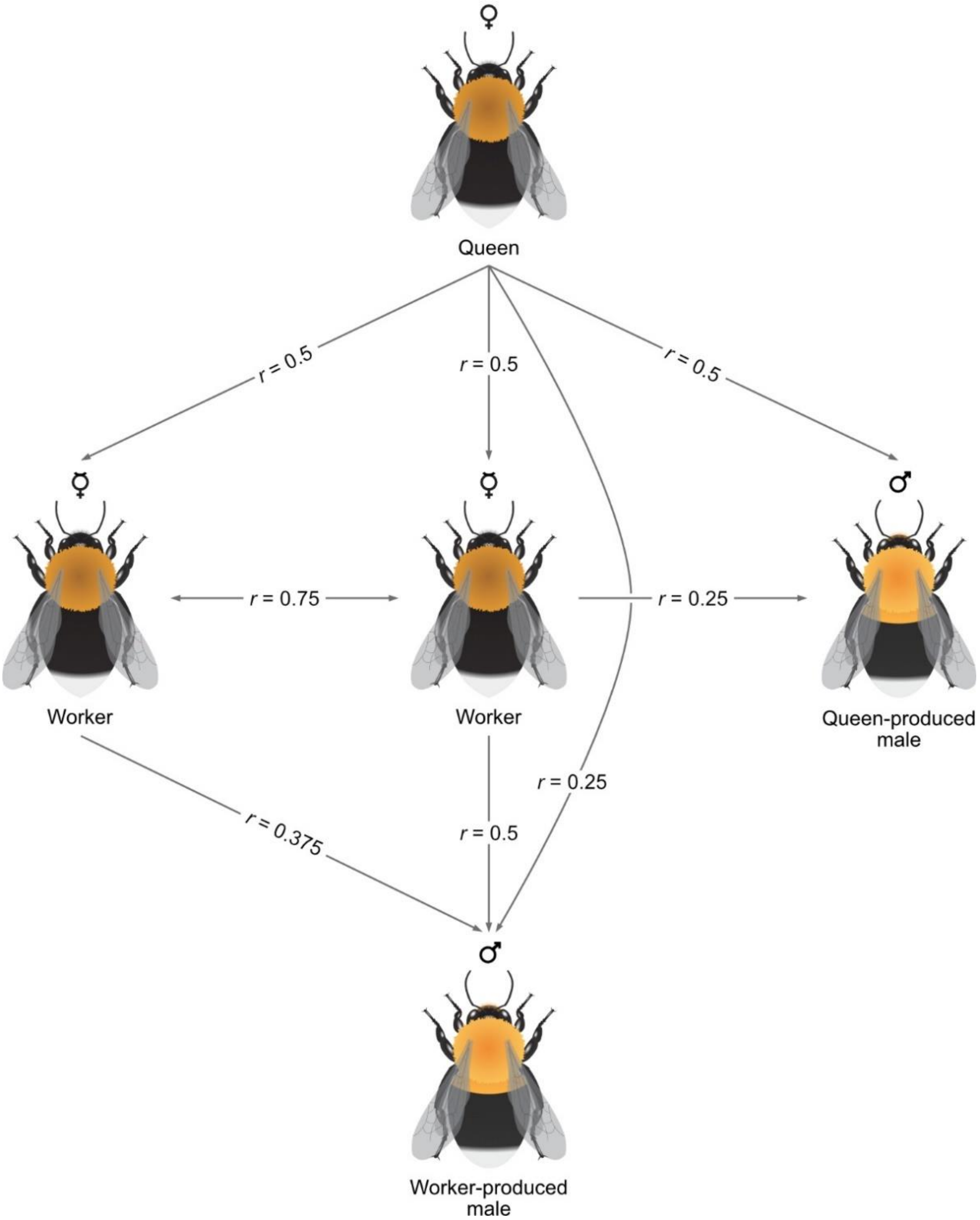

Figure 5.1. Within-colony relatedness (*r*) between colony members in a eusocial Hymenopteran colony headed by a single, monandrous queen. Haplodiploid sex determination leads to workers being more related to their own sons (*r* = 0.5) or their fellow workers' sons (*r* = 0.375) than to the queen's sons (*r* = 0.25), while queens are more related to their own sons (*r* = 0.5) than to their grandsons (*r* = 0.25), leading to conflict over male production between the queen and her workers.

Conflict therefore represents a major dissolutive force in social group formation and maintenance (Bourke, 2011a), and, as such, the evolutionary transition to eusociality in the Hymenoptera requires mechanisms that moderate within-group conflict over male production (Ratnieks & Reeve, 1992; Queller, 2000; Ratnieks & Wenseleers, 2008; Ratnieks & Helanterä, 2009; Ågren *et al.*, 2019). Male production conflict in eusocial Hymenopteran societies can be resolved through policing, a coercive mechanism that limits selfish worker reproduction via the queen and workers destroying worker-laid eggs and aggressing reproductive workers (Ratnieks, 1988; Ratnieks *et al.*, 2006; Ratnieks & Wenseleers, 2008; Ratnieks & Helanterä, 2009). Kin selection predicts that queens should favour policing, since, as mentioned, a queen is always more highly related to her own sons ($r = 0.5$) than to her workers' sons ($r = 0.25$; **Figure 5.1**; Hamilton, 1964b; Trivers & Hare, 1976). Indeed, queen policing is observed in species with relatively small colony sizes, such as ponerine and *Leptothorax* ants (Bourke, 1991; Nakata & Tsuji, 1996; Monnin & Peeters, 1997; Monnin & Ratnieks, 2001), *Polistes* paper wasps (Saigo & Tsuchida, 2004; Liebig *et al.*, 2005), yellow-jacket wasps (Wenseleers *et al.*, 2005a,b; Bonckaert *et al.*, 2011a), and at least one bumblebee species (*Bombus terrestris*; Zanette *et al.*, 2012).

However, queen policing may not always completely suppress worker reproduction. For instance, in the wasps *Dolichovespula sylvestris*, *P. chinensis*, and *Vespula rufa*, and the bumblebee *B. terrestris*, queens are responsible for eating 49-69% of all worker-laid eggs (Saigo & Tsuchida, 2004; Wenseleers *et al.*, 2005a,b; Zanette *et al.*, 2012). Further suppression of worker reproduction can therefore be achieved through workers mutually policing one another (Ratnieks, 1988; Ratnieks *et al.*, 2006; Ratnieks & Wenseleers, 2008; Ratnieks & Helanterä, 2009), and, in some species, queen and worker policing can act synergistically to resolve reproductive conflict (Wenseleers *et al.*, 2005b; Bonckaert *et al.*, 2011a; Zanette *et al.*, 2012).

Kin selection predicts that worker policing should occur when workers are more closely related to the queen's sons (brothers) than they are to their fellow workers' sons (nephews; Ratnieks, 1988; Crozier & Pamilo, 1996). In monogynous colonies, this situation arises when the effective queen mating frequency is higher than two, reducing worker-worker relatedness and causing worker relatedness to worker-produced males to fall below 0.25 (Ratnieks, 1988; Crozier & Pamilo, 1996). Accordingly, high rates of worker policing are observed in highly polyandrous species (Ratnieks & Visscher, 1989; Foster & Ratnieks, 2001b; Halling *et al.*, 2001; Oldroyd *et al.*, 2001; Bonckaert *et al.*, 2007), while comparative analyses across the eusocial Hymenoptera indicate low-levels of worker-produced males in species with low worker-worker relatedness (Wenseleers & Ratnieks, 2006). Additionally, effective worker policing may lower the survival probability of a worker-laid egg to such an extent that workers practice reproductive self-restraint, further reducing conflict within the colony (Ratnieks *et al.*, 2006).

To date, most studies analysing queen mating frequency and its effects on worker policing have focussed on across-taxon comparisons (Hammond & Keller, 2004; Wenseleers & Ratnieks, 2006). However, given separate taxa differ in ways besides kinship, such studies may be confounded by other variables that affect the outcome of male production conflict. Hence, species that exhibit facultative polyandry or facultative polygyny, leading to within-population, between-colony kin structure variation, are likely to represent the most powerful systems in which to investigate relationships between relatedness and reproductive conflict. Several such studies exist (Evans, 1998; Foster & Ratnieks, 2000; Foster *et al.*, 2001; Paxton *et al.*, 2001; Hammond *et al.*, 2003; Bourke, 2005; Helanterä & Sundström, 2007; Bonckaert *et al.*, 2011b), with the majority finding worker-produced males to be rare regardless of colony kin structure. Further, only two of these studies linked rates of worker egg laying (Hammond *et al.*, 2003) or policing (Foster & Ratnieks, 2000) to realised levels of worker reproduction, and so the generally low levels of worker male production observed could either be due to low levels of worker egg-laying or high levels of policing. Accordingly, relatedness-based predictions of intraspecific conflict resolution over male production in the eusocial Hymenoptera remain to be fully investigated.

Worker policing is also observed in a number of clonal or monogynous, monandrous species (Foster *et al.*, 2002; Hartmann *et al.*, 2003; Iwanishi *et al.*, 2003; Zanette *et al.*, 2012; Teseo *et al.*, 2013; Shimoji *et al.*, 2018), suggesting factors beside relatedness alone may influence worker policing. In such cases, worker policing may arise if policing of worker-laid male eggs allows the workers to produce a more female-biased sex ratio (Foster & Ratnieks, 2001c), or if it increases colony productivity, given colony-level costs of worker reproduction (Hartmann *et al.*, 2003; Teseo *et al.*, 2013). Alternatively, workers may direct their policing efforts towards reproductive workers if it increases their own chances of successfully reproducing (known as 'selfish policing'; Wenseleers *et al.*, 2005b; Stroeymeyt *et al.*, 2007; Bonckaert *et al.*, 2011a; Zanette *et al.*, 2012). Hence, many factors may select for worker policing, and act synergistically to reduce male production conflict, potentially increasing colony productivity. However, to date, interactions between within-population kin structure variation, levels of reproductive conflict, rates of policing, and colony productivity have yet to be thoroughly examined.

Bumblebees (*Bombus* spp.) comprise at least 250 obligately eusocial or socially parasitic species, with a natural distribution spanning the Americas, Eurasia and northern Africa (Williams *et al.*, 2008; Goulson, 2009). Generally, bumblebees exhibit annual colony cycles (**Figure 1.1**), with colonies consisting of a single, mated queen and tens to hundreds of her daughter workers (Alford, 2011). Colonies follow a general pattern of initial growth, during which the queen produces only workers, followed by a reproductive period, where the queen switches to producing gynes (virgin queens) and haploid males (Duchateau & Velthuis, 1989; **Figure 1.1**; **Figure 4.1**). Like most other eusocial Hymenopteran species, bumblebee workers are able to produce male offspring by laying unfertilised, haploid eggs (Bourke, 1988). In the well-studied species *B. terrestris*, workers begin to compete with the queen over male production (i.e. lay eggs and/or exhibit aggression) at a characteristic point in the colony cycle termed the 'competition point'

(Duchateau & Velthuis, 1988; Lopez-Vaamonde *et al.*, 2009), which generally follows the onset of sexual production. However, whether other *Bombus* species exhibit a clear-cut competition point is unknown. The realised level of adult male production by workers is highly variable across bumblebee species, with workers being responsible for as few as 4% of adult males in queenright (i.e. colonies with a queen) *B. terrestris* colonies (Lopez-Vaamonde *et al.*, 2004) and up to 85% of adult males in *B. wilmattae* (Huth-Schwarz *et al.*, 2011). However, apart from in *B. terrestris* (Zanette *et al.*, 2012), the behavioural processes underlying these conflict outcomes are largely unknown.

The Tree Bumblebee (*B. hypnorum*) is a widespread and abundant bumblebee across continental Europe and Asia that has recently undergone a westward range-expansion through the colonisation of the UK and Iceland from 2001 and 2008, respectively (Goulson & Williams, 2001; Prŷs-Jones *et al.*, 2016). Importantly, *B. hypnorum* exhibits facultative polyandry across both continental European and UK populations (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000; Paxton *et al.*, 2001; Brown *et al.*, 2002, 2003; Crowther *et al.*, 2019) and therefore provides a special opportunity in which to study relationships between relatedness, conflict resolution and colony productivity. Previous studies reported contrasting levels of worker male-production in European *B. hypnorum* populations (Paxton *et al.*, 2001; Brown *et al.*, 2003). For instance, Brown *et al.* (2003) found universal queen monandry in Swedish and Finnish populations, with workers accounting for over 20% of adult males in queenright colonies, suggesting that policing, if present, was not particularly effective. Conversely, Paxton *et al.* (2001) found an effective queen mating frequency of 1.26 in Swedish populations, with the queen accounting for all adult males, suggesting complete repression of selfish worker interests under queenright conditions. However, neither study carried out behavioural observations, and so nor did they quantify relative rates of egg-laying or policing carried out by the queen and her workers, which represent two important mechanisms underlying the resolution of queen-worker conflict in social insect colonies (Ratnieks *et al.*, 2006).

Therefore, the first aim of this study was to present the first quantitative behavioural and genetic analyses of male production conflict in queenright, unmanipulated *B. hypnorum* colonies, and the resulting proportions of queen- and worker-produced adult males. Besides being the first such study in *B. hypnorum*, this also represents the second such study across bumblebees, with detailed behavioural data only existing for *B. terrestris* (Zanette *et al.*, 2012). Obtaining data on policing mechanisms across a variety of taxa is therefore important to understand the origin and elaboration of conflict resolution from a phylogenetic context in the eusocial Hymenoptera. The second aim of this study was to relate levels of adult male production to colony kin structure and colony productivity, allowing a test of the hypotheses that worker policing occurs as a function of queen mating frequency and/or as a method of increasing colony productivity. I hypothesised that, as in other social insect species exhibiting annual colony cycles and relatively small colony sizes (Wenseleers *et al.*, 2005b; Zanette *et al.*, 2012), both queen and worker policing will occur regardless of colony kin structure but, in line with kin selection predictions, male production conflict will be reduced in polyandrous colonies, through either lower rates of worker egg-laying (i.e. reproductive self-restraint; Ratnieks *et al.*, 2006) or higher rates of worker policing. Further, I hypothesise that lower colony-level male production conflict will lead to higher colony productivity.

5.3. Methods

(a) Colony rearing and parasite screening

Twenty-eight *B. hypnorum* colonies (n_{2018} = 8; n_{2019} = 20) were reared from two cohorts of queens (*n* = 263) collected from the field between 7 March and 19 April 2018 and 25 February and 9 April 2019. The 2018 queen cohort (*n* = 107) was collected from Great Windsor Park, Surrey (*n* = 25), Chingford, Greater London (*n* = 7), and various sites across Norwich, Norfolk (*n* = 75). The 2019 queen cohort (*n* = 156) was collected at various sites across Norwich, Norfolk. Queens bearing pollen on their corbiculae were not collected, since it is likely that such queens have already founded nests and will not found nests in captivity (Tripodi & Strange, 2019). Previous research (Huml *et al.*, 2021) found no evidence of genetic differentiation between UK subpopulations of *B. hypnorum*. Hence, all queens collected in the present study were considered representative of the UK population and pooled.

Queens were reared in custom-made acrylic queen rearing boxes (dimensions: L14 × W8 × D5 cm; Signforce Ltd, Norwich) under constant conditions (mean ± range: 27°C ± 1°C; 60% ± 10% RH) and provided with pollen (Sussex Wholefoods, Eastbourne, UK) and 50% Apiinvert sugar syrup (Südzucker AG, Mannheim, Germany) *ad libitum*. Once 10-20 workers had eclosed, the queen, workers, and brood (eggs, larvae, and pupae) were transferred into wooden nest boxes (dimensions: L30 × W20 × D17 cm), with pollen and sugar syrup continuing to be provided *ad libitum*.

As part of a separate study (**Chapter 3**), the infection status of all collected queens (including each of the 28 colonies used in the present study) was determined via the screening of queen faecal samples under an Olympus BX41 phase-contrast microscope (Olympus Corp., Tokyo, Japan) at 100-400× magnification. Queen faeces were checked for four generalist bumblebee endoparasites known to infect *B. hypnorum* in the UK population (Jones & Brown, 2014): (1) the neogregarine *Apicystis bombi*; (2) the trypanosome *Crithidia bombi*; (3) the microsporidian *Nosema bombi*; and (4) the nematode *Sphaerularia bombi* (**Figure 3.2**). Each queen from the 2018 cohort was screened within 24 hours of collection and once again 7-10 days post-collection. Given that *S. bombi* larvae were never found during the faecal screenings of 2018 queens (**Chapter 3**), the period between the first and second faecal parasite screening was extended for the 2019 queens. Accordingly, each queen from the 2019 cohort was screened within 24 hours of collection and once again 21 days post-collection. Infected queens/colonies were defined as those in which one or more of the four parasite species that were screened for was found, and uninfected queens/colonies were defined as those in which none of these four parasites was found. To ensure cross-contamination did not occur between infected colonies, infected and uninfected colonies were kept separate from one another, and different handling equipment was used for the two classes of colony. Further, handling equipment was sterilised in between the handling of individual colonies across both infected and uninfected groups. Besides this, infected queens and their colonies were treated identically to uninfected queens and their colonies (i.e. colonies were reared from queens regardless of whether they were infected).

S. bombi eggs and larvae did not appear to be present in queen faeces even in infected queens (**Chapter 3**), and were not found during faecal parasite screenings. Hence, queen infection with *S. bombi* was confirmed by the post-death dissection of queens to check for the presence of adult *S. bombi* in the abdominal cavity under a Zeiss Discovery v12 stereomicroscope (Zeiss, Oberkochen, Germany) at 10-30× magnification. Note that, since *S. bombi* infection could not be reliably inferred through faecal screening, *S. bombi*-infected queens would have initially been falsely considered as uninfected and reared alongside other genuinely uninfected queens. However, there would have been no chance of *S. bombi* cross-infection given that handling equipment was sterilised in between handling of different colonies, and that *S. bombi* females can only infect queens during the overwintering stage of the bumblebee life-cycle (Poinar Jr & Van der Laan, 1972).

(b) Colony demography and productivity

Colonies were checked daily for production of adult offspring and for the presence of individuals (queens or workers) that had died. All adult workers were individually marked on the day of eclosion with numbered discs (EH Thorne Ltd, Market Rasen, UK). For each colony, the day of first worker eclosion was designated as colony day one. All adult sexuals (males and gynes) were removed on the day of eclosion and frozen at -20˚C, since, in nature, adult sexuals are known to disperse from the nest a few days after eclosion (Alford, 2011). Upon death, the colony queen and workers were removed and frozen at -20˚C, and the death date was recorded for each individual. Worker longevity was defined as the time in days between eclosion and death. Queen longevity was defined as the time in days between colony day one and her death. Colony longevity was defined as the time in days between first worker eclosion and last offspring eclosion.

To determine the population-level per capita mean mass of males and gynes, eight whole individuals were selected at random from each colony and dried at 60˚C for four days before being weighed individually to the nearest 0.1 mg (BDH Balance Model 100A, Milton Keynes, UK). In those colonies that did not produce at least eight of each class of individual, as many as possible of each sex and caste were weighed. A total of 224 males ($n_{colonies}$ = 28) and 185 gynes ($n_{colonies}$ = 26) were used to calculate the population mean mass of males and gynes. Mean ± SD dry masses of males and gynes were 48.5 ± 13.4 mg and 135.5 ± 25.9 mg, respectively. Population-level mean male and gyne dry masses were used to convert numerical sex ratios into sex investment ratios, defined as total female dry mass divided by total sexual dry mass produced, for each colony.

(c) Behavioural observations

Because it was unknown whether a clear-cut competition point occurs in *B. hypnorum* (see **Introduction**), behavioural observations were started once colonies contained 20 or more workers or had produced at least one sexual (mean ± SD of workers at start of behavioural observations = 24.8 ± 4.0 workers; mean ± SD day of behavioural observation start = 25.8 ± 7.6).

Behavioural data were collected between 09:00 and 20:00, five days per week (Monday-Friday) from 3 May to 3 July 2018 and from 3 April to 12 July 2019. Behavioural data consisted of hour-long recordings of each colony using a high-definition camcorder (Sony HDR-CX190E) under white light (mean ± SD film observation length = 63.5 ± 5.0 minutes). The camera was placed directly above the colony, with a field of view covering the entire brood (the wax nest structure containing all juvenile developmental stages). Hence, all behaviours taking place between individuals on the nest itself were observable. The white light used for recordings had no obvious effect on bee behaviour. The 2019 colony cohort were also subjected to 5-minute direct observations five days per week (Monday-Friday), with the following behaviours recorded: (1) queen or worker egg-laying; (2) queen or worker egg-eating; and (3) queen-worker (i.e. aggression directed from the queen towards a worker and vice versa) and worker-worker aggression in the form of darting or attack (Duchateau, 1989). Darting was defined as an aggressive individual making a sudden movement towards another individual but not making contact, while attack was defined by an aggressive individual stinging and/or biting another individual (Duchateau, 1989). When either queen or worker egg-laying was observed during the direct observations, a camcorder was set up to record the egg-cell in which laying had been observed for up to two hours post-laying, allowing the determination of egg survival (Zanette *et al.*, 2012). Further, any egg-laying events that were observed during colony maintenance (e.g. worker marking, sexual removal, colony cleaning etc.) were also subjected to recording for up to two hours. Finally, since the camcorder’s field of view during filming of focal egg-cells was usually larger than a single egg-cell, egg-laying events were often observed during the retrospective viewing of egg-cell recordings, and data from these egg-laying events were also collected. Where possible, the number of eggs laid or eaten by the queen or worker per egg-laying/egg-eating event was also recorded. However, this measure was often prevented due to the egg-laying/egg-eating individual’s body or head obscuring the observer's view of the eggs. For all observations, the identities of the actor and recipient of these behaviours were recorded (from the numbered disks) where possible.

Both egg-laying and egg-eating events were easily detectable due to the characteristic behaviours observed before, during and after each event. In the case of egg-laying, individuals would construct and adjust the egg-cell with their mandibles until it fitted their abdomen tip, leading to a characteristic spinning behaviour prior to egg-laying. Laying individuals then inserted their abdomen tip into the open egg-cell, often with the sting extruded, and remained stationary for several minutes as they laid eggs (**Figure 5.2A-B**). Following successful egg-laying, the egg-layer would then spend several minutes waxing over the egg-cell using their mandibles, resulting in a closed egg-cell containing eggs. In the case of egg-eating, individuals exhibited a rocking motion of the head as they opened egg-cells with their mandibles. Once the egg-cell was open, the individual placed its head inside it, pulled out the eggs inside with its mandibles, and consumed them (**Figure 5.2C-D**), resulting in a previously closed egg-cell becoming open and empty.

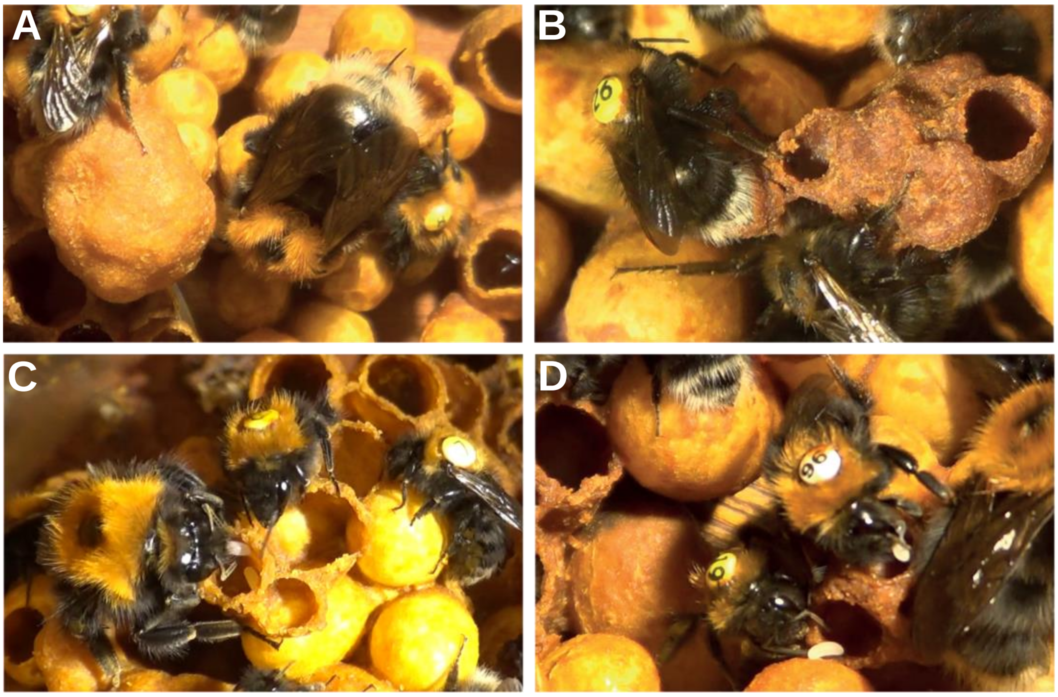

Figure 5.2. Queen-worker conflict over male parentage in Tree Bumblebee (*Bombus hypnorum*) colonies. **(A)** Colony queen and **(B)** worker #26 laying eggs. Note the placement of the abdomen tip inside the open egg-cell and, in the queen's case, the extruded sting, characteristic of bumblebee egg-laying. **(C)** Colony queen eating the first of two eggs eaten, which were laid by one of her daughter workers (seen egg-laying into the cell earlier). **(D)** Workers #19 and #196 eating two eggs laid by one of their sister workers (again, seen egg-laying into the cell earlier). All images are stills taken from digital film recorded using a Sony HDR-CX190E camcorder.

For each colony, the competition point was defined as the first day on which worker-egg laying or queen or worker egg-eating was observed. In two colonies (colony 216 and 324), such events were never observed, and so the competition point for these colonies was defined as the date of first observed queen-worker aggression. Behavioural data on rates of egg-laying, egg-eating and egg survivorship were only analysed for queenright, post-competition point (henceforth referred to as 'competition phase') colonies. In a single colony (colony 94), the queen died one day after the onset of the competition point. Therefore, this colony was excluded from observational data analysis. The competition phase observation period per colony lasted for a mean ± SD (range) of 27.15 ± 11.34 (7-45) days, resulting in a mean ± SD (range) of 18.70 ± 7.67 (5-32) days of observational data per colony, and a mean ± SD of 19.8 ± 8.1 hours of observational recordings per colony.

Per-hour rates of egg-laying and egg-eating by queens and workers were calculated for each colony from the hour-long digital recordings by multiplying the number of egg-laying and egg-eating events observed by the mean number of eggs laid or eaten by each caste, before dividing this value by the digital film length. Given that the camera's field of view covered the entire brood mass and that the queen was always on the brood during recordings, these calculations would not have been biased by off-screen laying events (i.e. those outside of the camera's field of view). The number of worker-laid eggs left surviving per hour was then calculated by subtracting the number of worker-laid eggs eaten (by either the queen or the workers) from the number of worker eggs laid.

Survival frequency of queen- and worker-laid eggs was analysed by retrospective viewing of the focal egg-cell videos, with egg survival time defined as the time in minutes between the end of the egg-laying event until the eating of the focal eggs or until the video ended (with such eggs classed as surviving). Laying individuals were often observed being attacked and pulled off the egg-cell during egg-laying, and so each egg-laying event was classified as either interrupted or uninterrupted. All egg-laying events and their subsequent survivorship observed during the hour-long behavioural videos were also included as datapoints in the egg survival analysis.

All filmed observational data were analysed using the software package BORIS v7.9.15 (Friard & Gamba, 2016) at 0.5-8.0× playback speed. All behavioural data were collected and analysed blind to queen mating frequency and worker-worker relatedness.

(d) Queen mating frequency and worker-worker relatedness

To estimate queen mating frequency and worker-worker relatedness, the queen and a random sample of 15 workers from each colony were genotyped at 20 polymorphic microsatellite loci as previously deployed in Crowther *et al.* (2019). In two colonies from the 2019 cohort, the queen genotype was unavailable due to non-recovery of the queen. In these cases, the queen genotype was reconstructed from the genotypes of the first 8 males to eclose within each colony (since such males will all be queen-produced).

DNA was salt-extracted from the flight muscle tissue of the sampled queens and workers using an ammonium-acetate ethanol precipitation procedure modified from Richardson *et al.* (2001). To maximise DNA yield, the ethanol precipitation step included storage at -20˚C for at least four hours. Final DNA extractions were suspended in low EDTA-TE buffer (10 mM Tris.HCl, 0.01 mM EDTA) and the quality of extractions was checked using a Nanodrop 8000 (Thermo Fisher Scientific, UK).

All PCRs were carried out following the method outlined in Crowther *et al.* (2019), with all samples being genotyped at 20 microsatellite loci (Estoup *et al.*, 1995; Reber Funk *et al.*, 2006; Stolle *et al.*, 2009), across three primer multiplexes. Each PCR plate included: (1) a negative control, consisting of all reagents and primers but no template DNA; and (2) a positive control in the form of DNA extracted from a haploid male, whose genotype was known from previous characterisation (**Chapter 2**).

PCR products were visualised using a 48-well capillary ABI 3730 DNA analyser and a ROX-500 internal size standard (Thermo Fisher Scientific, UK), and peaks were determined using GeneMapper 4.0 (Thermo Fisher Scientific, UK) software. All positive control genotypes matched up to the previously characterised male genotype, and none of the negative controls showed any peaks corresponding to the amplified alleles. As in **Chapter 2**, the BTMS0132 locus was found to be monomorphic and was not used in any further analyses. Across all colonies, a total of 420 workers (n_{2018} = 120; n_{2019} = 300) and 28 queens (n_{2018} = 8; n_{2018} = 20 [two reconstructed from male genotypes]) were genotyped at a median (range) of 18 (7-19) loci.

Colony v2.0.6.6 (Jones & Wang, 2010) was used to determine the maximum likelihood paternity assignments for the 420 workers sampled from the 28 colonies. Genotypes for the 19 loci were input for queens and workers, along with maternity relationships (which queens were the mothers of which workers), maternal sibships (which workers were maternal sisters) and maternal sibship exclusions (which workers could not be maternal sisters). All these relationships could be determined since each colony was kept in an individual colony box, with no intermixing of workers between colonies. Given previous studies on facultative polyandry in *B. hypnorum* (Estoup *et al.*, 1995; Paxton *et al.*, 2001; Brown *et al.*, 2003; Crowther *et al.*, 2019), the female mating system was specified as polygamous. Given the highly male-biased sex ratios found in bumblebee populations (Bourke, 1997), including *B. hypnorum* (this study; **Chapter 4**), it is extremely unlikely for a single male to mate with more than one queen and hence the male mating system was specified as monogamous. Each analysis used a single long full-likelihood run, with allele frequencies updated during the run, sibship scaling, and a weak sibship size prior of 8.6 siblings per patriline (based on dividing the per colony worker sample size by the mean queen mating frequency of 1.7 found in Crowther *et al.* (2019)) and 15 siblings per matriline. Paternity analyses were run with and without inbreeding, and with genotyping error rates of 0.05 and 0.01. All four runs gave the same patriline assignments to all workers, and all workers were assigned to the correct matriline (i.e. no mismatches were found between queen and worker genotypes within each colony). Worker-worker relatedness was calculated for each colony by running 100 iterations of the Wang full-likelihood pairwise-relatedness estimator (Wang, 2002) in the *related* R package (Pew *et al.*, 2015). The 420 worker genotypes were also used to calculate allelic richness and observed and expected heterozygosity using CERVUS v3.0.7 (Kalinowski *et al.*, 2007).

Numeric queen mating frequency was estimated from the number of patrilines identified across the sampled workers in each colony. Effective queen mating frequency (k_e) was estimated from the paternity share observed across the sampled workers in each colony, using the following sampling bias-corrected formula from Nielsen *et al.* (2003):

$$k_e = \frac{(n-1)^2}{\sum_{i=1}^{k_0} \left[\hat{p}_i^{\,2}\,(n+1)(n-2)\right] + 3 - n} \qquad \textbf{Equation 5.1}$$

where n equals the number of sampled workers per colony, k_o is the observed number of patrilines across the sampled workers, i indexes each individual patriline identified within the colony, and $\hat{p}_i$ equals the proportion of the sampled workers sired by each observed patriline.

Three types of error may confound estimates of effective paternity in eusocial Hymenoptera (Boomsma & Ratnieks, 1996). Firstly, patrilines within a colony may go undetected due to males sharing identical genotypes across the microsatellite markers under investigation, leading to daughter workers from different patrilines being indistinguishable from one another. Non-detection error (D_p) was calculated from Boomsma & Ratnieks (1996) as the probability that two paternal males had identical genotypes at all 19 microsatellite as:

$$D_p = \prod\left(\sum q_i^{\,2}\right) \qquad \textbf{Equation 5.2}$$

where $q_i$represents the frequency of the ith allele, summed over all unique alleles at a locus, and multiplication is across all loci.

Secondly, paternity skew in social insects may lead to some patrilines going undetected due to non-sampling of a male's offspring. The probability of such non-sampling error (S_p) was calculated from Boomsma & Ratnieks (1996) as:

$$S_p = 1 - (1 - p_s)^n$$ **Equation 5.3**

where p_s represents the proportion of offspring sired by a second patriline and n represents the number of worker offspring sampled.

Finally, non-detection of multiple patrilines may be caused by temporal variation in sperm usage, under which a multiply-mated queen fertilises early workers with sperm from one patriline, and later workers with sperm from another (Boomsma & Ratnieks, 1996). To account for this, workers eclosing across the entire worker-production period for each colony were randomly sampled for genotyping.

(e) Male genotyping and parentage analysis

Previous research suggests that developmental time (egg to adult eclosion) lasts 18 days for *B. hypnorum* males (Röseler & Röseler, 1974). Hence, to estimate realised levels of worker male production, a sample of 32 adult males that eclosed 20 days or more after the onset of the competition point, and no more than 18 days post-queen death, were randomly selected for genotyping and parentage assignment. Accordingly, the sampled males would have developed from eggs laid under competition phase, queenright conditions when both the queen and her workers were laying male-destined eggs (with the queen laying ~77% haploid eggs at this stage; **Chapter 4**). In seven of the reared *B. hypnorum* colonies, fewer than 32 males eclosed 20 days after the onset of the competition point, and so adult males were sampled for 21 of the 28 colonies.

Genotyping of the queen and her workers allowed the identification of informative loci for male parentage assignment in each colony (i.e. assignment as queen- or worker-produced males), with informative loci defined as those loci at which the genotypes included alleles present in workers but not the queen (i.e. paternal alleles). Once informative loci were identified, males were then genotyped across 1-2 of the microsatellite multiplexes, at a median (range) of 3 (2-6) informative loci, to assign parentage. Males were assigned as worker-produced if they exhibited one or more of the paternal alleles known to be present in each colony. DNA extractions, PCRs and allele peak visualisation were carried out as for the queens and workers above, with 672 adult males being genotyped in total. There was no overlap between the adult males genotyped in the current study and those genotyped in **Chapter 2**.

Finally, all 26 initially genotyped queens and a subset of 3-11 males sampled for parentage assignment from each colony were regenotyped to determine genotyping error rate at each microsatellite locus. Therefore, a range of 7.37-11.69% of all reactions across the three microsatellite panels were subject to regenotyping. From regenotyping samples, the per-locus mean (range) mistyping rate was 0.72% (0.00-4.30%; **Table 5.1**).

(f) Statistical analyses

All statistical analyses were carried out using R v4.0.1 (R Core Team, 2020), with all data visualised using the *ggplot2* R package (Wickham, 2009).

Two-sample Wilcoxon tests were used to test differences in the number of eggs laid per egg-laying event between queens and workers, between workers that were interrupted and workers that were uninterrupted during egg-laying, and between the expected and observed frequencies of worker-produced adult males. Expected frequencies of worker-produced males were calculated for each colony by multiplying the proportion of eggs laid by workers eggs during the competition phase by their chance of survival (calculated from focal egg-cell recordings) and dividing this value by the proportion of surviving queen- and worker-laid eggs. Fisher's exact tests were used to test for associations between worker policing (via attack or egg-eating) and worker egg-laying.

Table 5.1. Genetic diversity and genotyping error rate data for each of the 19 microsatellite loci at which *Bombus hypnorum* queens, workers and males were genotyped at ($n_{genotyped\ individuals}$ = 1120). Locus, name of each microsatellite locus; N_A, number of unique alleles found at each microsatellite locus; error rate, the error rate from mistyping at each microsatellite locus; H_o, observed heterozygosity at each microsatellite locus; H_e, expected heterozygosity at each microsatellite locus. Alleles found at each locus are then numbered 1-8 in ascending order of base pair size: Length, the base pair length of each unique allele; Freq., the frequency of each unique allele.

					Allele 1		Allele 2		Allele 3		Allele 4		Allele 5		Allele 6		Allele 7		Allele 8	
Locus	**N_A**	**Error rate**	**H_o**	**H_e**	Length	Freq.	Length	Freq.	Length	Freq.	Length	Freq.	Length	Freq.	Length	Freq.	Length	Freq.	Length	Freq.
B131	3	0.021	0.323	0.359	118	0.040	120	0.779	127	0.182										
BL03	5	0.043	0.499	0.511	144	0.350	146	0.005	148	0.030	150	0.605	156	0.010						
BT26	4	0.000	0.486	0.483	97	0.045	101	0.694	103	0.125	110	0.137								
B132	5	0.000	0.462	0.461	159	0.205	171	0.045	172	0.704	173	0.028	178	0.019						
BTMS0125	8	0.000	0.714	0.745	110	0.081	113	0.230	125	0.408	132	0.018	137	0.133	144	0.106	148	0.016	149	0.007
B11	4	0.009	0.674	0.642	156	0.001	158	0.288	162	0.463	164	0.248								
B10	6	0.000	0.573	0.636	191	0.517	192	0.022	193	0.123	194	0.045	199	0.010	200	0.284				
B96	3	0.009	0.632	0.645	251	0.230	253	0.328	255	0.442										
BTMS0057	5	0.018	0.748	0.700	104	0.031	107	0.166	109	0.436	111	0.096	113	0.270						
BT05	4	0.018	0.747	0.651	153	0.007	156	0.447	158	0.248	162	0.298								
BTMS0033	2	0.018	0.286	0.329	201	0.214	204	0.787												
BTMS0056	3	0.000	0.207	0.206	254	0.041	256	0.888	257	0.071										
B121	5	0.000	0.708	0.707	153	0.142	159	0.028	168	0.377	170	0.347	208	0.106						
BT10	2	0.000	0.460	0.414	118	0.707	121	0.293												
BTERN02	7	0.000	0.708	0.701	157	0.067	163	0.482	165	0.172	167	0.065	169	0.028	175	0.020	179	0.168		
BTMS0083	6	0.000	0.548	0.694	277	0.239	279	0.049	294	0.018	302	0.262	304	0.010	306	0.422				
BTERN01	3	0.000	0.141	0.166	114	0.910	116	0.014	125	0.076										
BL08	2	0.000	0.165	0.184	145	0.898	149	0.102												
BL01	4	0.000	0.560	0.558	137	0.600	141	0.261	142	0.118	148	0.021								

Kaplan-Meier survival curves (accounting for censoring) and log-rank tests were carried out using the *survival* R package (Therneau, 2020), to test survivorship differences between queen- and worker-laid eggs and between the longevities of egg-laying and non-egg laying workers.

Two models were produced to determine the potential impacts of conflict resolution on overall colony productivity. Firstly, the total sexual biomass produced per colony (in grams) was fitted as the response in a generalised linear model with Gaussian distribution and a log link function. Secondly, the colony sex ratio was fitted as the response in a beta regression model with a logit link function, fitted using the *betareg* R package (Cribari-Neto & Zeileis, 2010). For both models, the year (2018 vs 2019), infection status (uninfected vs infected), total number of workers, queen lifespan, colony lifespan, percent of egg-laying workers, hourly rate of worker egg-laying, hourly rate of egg-eating carried out by the queen, and worker-worker relatedness were included as predictor variables.

For both models, the maximal model including all predictors was initially fitted. Minimal adequate models were then selected from the maximal models using the *dredge()* function in the *MuMIn* R package (Barton, 2020), which selects models by searching through all possible predictor combinations and reporting values of the corrected Akaike information criterion accounting for small sample sizes (AIC_c; Hurvich & Tsai, 1993). The candidate model with the lowest AIC_c was then kept and reported as the best model. The adjusted R^2 value was calculated for the sexual productivity model using the *rsq* R package (Zhang, 2017, 2021). The pseudo R^2 value was calculated for the sex ratio model using the *betareg* R package (Cribari-Neto & Zeileis, 2010). Model assumptions were visually assessed using the *autoplot()* function from the *ggfortify* R package (Tang *et al.*, 2016).

All means are presented ± 1 standard deviation unless otherwise stated.

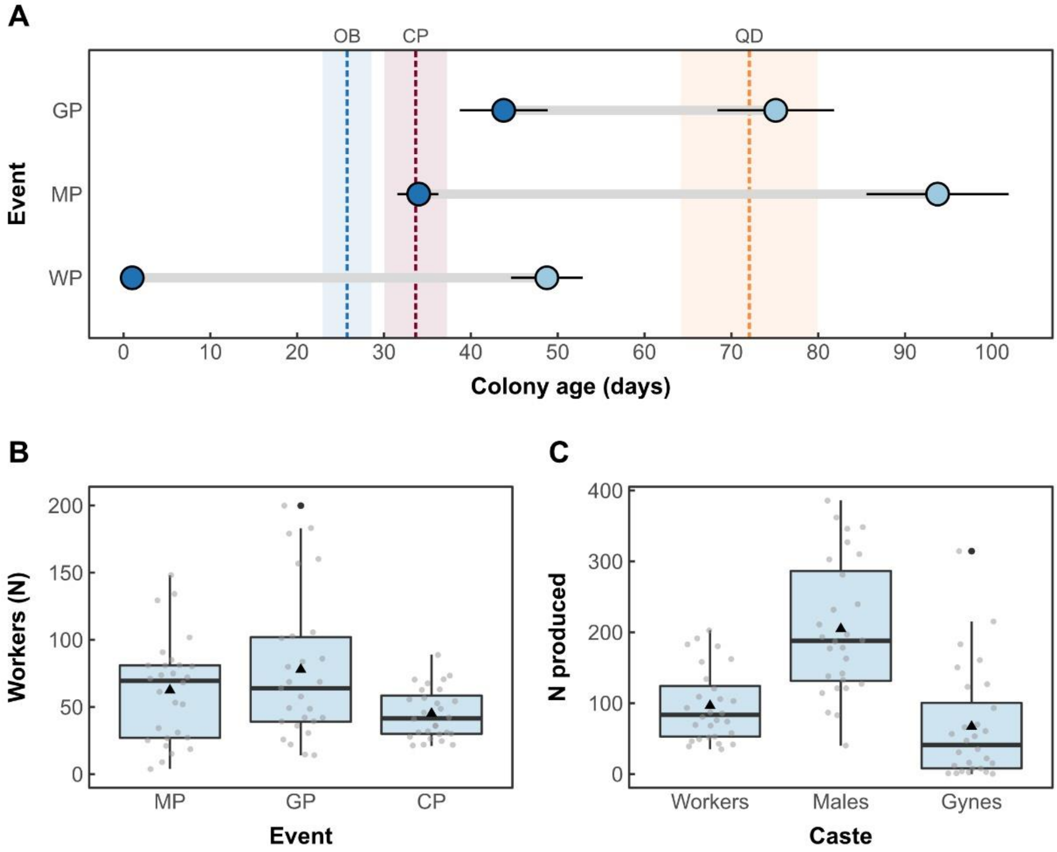

Figure 5.3. Event timings and colony demography for 28 Tree Bumblebee (*Bombus hypnorum*) colonies. **(A)** Mean start (dark blue circles) and end points (light blue circles) with 95% confidence intervals (whiskers) of worker, male and gyne production (‘WP’, ‘MP’ and ‘GP’, respectively) throughout the colony cycle. Note there are no confidence intervals for the start of worker production since the day of first worker eclosion represented day 1 for every colony. Dashed blue line, mean start point of behavioural observations (‘OB’); dashed burgundy line, mean start point of the competition point (‘CP’); dashed orange line, mean day of queen death (‘QD’); shaded area around each dashed line, 95% confidence interval of event timing. **(B)** Number of workers within each colony at the onset of male production (first male eclosion), gyne production (first gyne eclosion) and the competition point (first observation of worker egg-laying or queen-worker aggression). **(C)** Total number of workers, males and gynes produced by each colony. For boxplots **(B)** and **(C)**: thick horizontal bars, median; triangles, mean; boxes, interquartile range (IQR); whiskers, range (not including outliers); filled black circles, outliers, defined as points more than 1.5 IQR above or below the upper or lower quartile; filled grey circles, ‘jittered’ raw data, with each point representing a single colony.

5.4. Results

(a) Parasite infection, colony demography, and productivity

Parasite screening revealed that 23 of the colony queens were uninfected, four colony queens were infected with *C. bombi* and one colony queen had a dual infection of *C. bombi* and *S. bombi*.

Across the 28 colonies, there were a mean of 24.1 ± 3.7 days between queen collection and the onset of worker production (defined by the eclosion of the first worker). Mean colony lifespan was 94.2 ± 21.7 days and mean queen lifespan was 72.1 ± 21.3 days (**Figure 5.3A**). Colonies produced a mean of 96.5 ± 51.3 workers (**Figure 5.3C**) across the entire colony cycle, with the worker production period lasting a mean of 48.8 ± 11.2 days (**Figure 5.3A**).

Males were produced by all 28 colonies, with a mean of 204.9 ± 94.9 males produced per colony (**Figure 5.3C**). On average, male production began on day 33.9 ± 6.4 (range: 20-49) of the colony cycle and the male production period lasted for a mean of 60.9 ± 22.6 days (**Figure 5.3A**). Colonies contained a mean of 62.0 ± 50.0 workers at the onset of male production (**Figure 5.3C**).

Gynes were produced by 27 of the 28 colonies, with a mean of 67.1 ± 78.3 gynes produced per colony (**Figure 5.3C**). On average, gyne production began on day 43.8 ± 13.4 of the colony cycle (range: 22-65 days) and the gyne production period lasted for a mean of 32.3 ± 21.7 days (**Figure 5.3A**). Colonies contained a mean of 77.9 ± 54.4 workers at the onset of gyne production (**Figure 5.3B**).

(b) Egg-laying, egg-eating, and male parentage

On average, the competition point occurred on day 33.6 ± 9.7 (range: 21-57) of the colony cycle, with behavioural observations beginning 7.9 ± 6.4 days prior to the competition point (**Figure 5.3A**). Hence, *B. hypnorum* appears to exhibit a clear-cut competition point, given that there was approximately a week between the onset of behavioural observations (with no queen-worker aggression, egg-eating, or worker egg-laying observed during this time) and the competition point (**Figure 5.3A**). Colonies contained 45.2 ± 18.8 workers on the date of the competition point (**Figure 5.3B**).

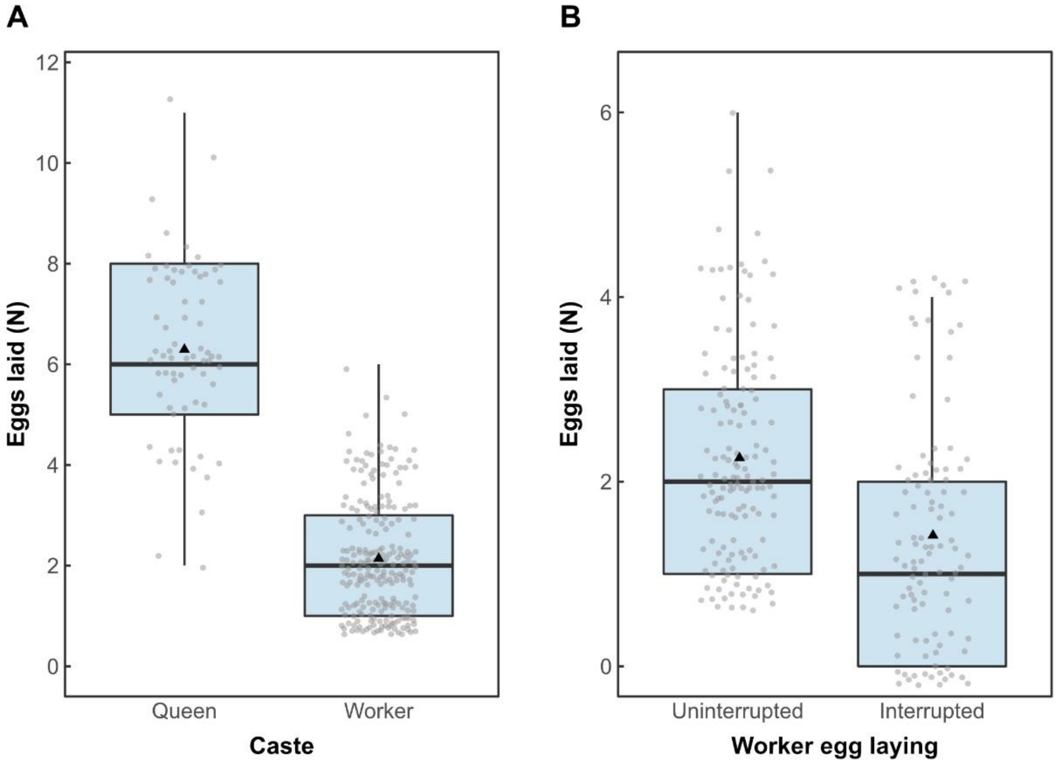

Figure 5.4. Number of eggs laid per egg-laying event across 27 Tree Bumblebee (*Bombus hypnorum*) colonies. **(A)** Number of eggs laid by queens and workers per successful egg-laying event ($n_{queen\ events}$ = 68; $n_{worker\ events}$ = 220). **(B)** Number of eggs laid by workers per egg-laying event (successful and unsuccessful) depending on whether the reproductive worker was attacked and pulled off the egg-cell (interrupted) during egg laying ($n_{uninterrupted\ events}$ = 144; $n_{interrupted\ events}$ = 103). Thick horizontal bars, median; triangles, mean; boxes, interquartile range (IQR); whiskers, range; filled grey circles, 'jittered' raw data with each point representing a single egg-laying event. Note that data came from 27 of the 28 colonies in the present study, since in a single colony (colony 94), the queen died one day after the onset of the competition point. This colony was therefore not used for behavioural observations.

Across all behavioural observations (direct observations plus hour-long behaviour and egg survival videos), a total of 643 egg-laying events were observed. Queens accounted for 126 (19.6%) of all observed egg-laying events, and workers accounted for the remaining 517 (80.4%). Queen egg-laying was observed in 24 of the 27 colonies (88.9%) for which post-competition point behavioural data were available, with 4.7 ± 4.5 queen egg-laying events observed per colony. Worker egg-laying was observed in 25 of the 27 colonies (92.6%), with 19.2 ± 22.8 worker egg-laying events observed per colony. A total of 203 unique workers were observed laying eggs, with each colony containing 7.5 ± 6.1 egg-laying workers, constituting 7.6% ± 5.6% of all workers.

Eggs were successfully laid (i.e. at least one egg was laid) in 616 of the 643 (95.8%) observed egg-laying events, with 126 and 490 successful queen-and worker-laying events, respectively. The number of eggs laid could be counted in 68 (53.97%) and 220 (45.01%) of the successful queen- and worker-laying events, respectively. Queens laid a significantly larger number of eggs per egg-laying event (6.29 ± 1.77) than workers (2.14 ± 1.11) (Wilcoxon rank sum test: W = 14,498, $p < 0.001$; **Figure 5.4A**). During the competition phase, *B. hypnorum* queens laid 0.57 ± 0.59 eggs per colony per hour, while workers collectively laid 1.02 ± 1.02 eggs per colony per hour (**Figure 5.5A**). Therefore, on average, workers accounted for 64.2% of all eggs laid during the competition phase.

A total of 328 egg-eating events were observed overall ($n_{queen} = 318$; $n_{worker} = 10$), and the number of eggs eaten per event could be counted for 152 (47.80%) and 7 (70.00%) of the queen and worker egg-eating events, respectively. Queens and workers ate 2.17 ± 1.13 and 1.14 ± 0.38 eggs per policing event, respectively. During the competition phase, queens and workers ate 0.65 ± 0.72 and <0.01 ± 0.01 worker-laid eggs per colony per hour, respectively (**Figure 5.5B**), leading to an estimated 0.37 ± 0.45 worker-laid eggs surviving per hour (**Figure 5.5C**).

Egg-laying was often associated with aggressive behaviour. In 3 of 126 (2.4%) observed queen-laying events, egg-laying was interrupted due to the queen being attacked and pulled off the egg-cell by one of her workers. In 143 (27.7%) and 27 (5.2%) of all 517 worker-egg laying events, egg-laying was interrupted due to the queen or another worker, respectively, attacking and pulling the laying worker off the egg-cell. In 27 (5.2%) of the 517 worker-egg laying events, queen or worker interruption prevented the laying worker from laying any eggs, with queens being responsible for 25 of these attacks (92.6%) and workers for the remaining two (7.4%). The number of eggs laid could be counted in 144 (41.5%) and 103 (60.6%) of the 347 uninterrupted and 170 interrupted worker-laying events, respectively. Workers that were uninterrupted during egg laying laid significantly more eggs per egg-laying event (2.3 ± 1.1) than workers that were interrupted during egg laying (1.4 ± 1.3) (Wilcoxon rank sum test: W = 10,389, $p < 0.001$; **Figure 5.4B**).

The survival of eggs in 119 (94.4%) and 474 (96.7%) of the 126 and 490 successful queen and worker egg-laying events, respectively, could be tracked for up to two hours. A total of 324 egg-eating events were observed, with the eating of worker-laid eggs accounting for 323 (99.7%) of these observations. In the single case of queen-laid eggs being eaten, the queen laid six eggs, two of which were eaten by a worker 108 minutes after the eggs had been laid (**Figure 5.6**). Accounting for censored data (i.e. where an egg could not be tracked for the entire two hours), survival analysis revealed that 99.6% of queen-laid and 23.4% of worker-laid eggs remained two hours after being laid, with half of all worker-laid eggs having been eaten within 11.5 minutes of being laid (**Figure 5.6**). Worker-laid eggs were therefore significantly less likely to survive than queen-laid eggs (Log rank test: $\chi^2 = 150$, df = 1, $p < 0.001$).

Queens were responsible for 315 (97.5%) of the worker-laid egg eating events, workers were responsible for 5 (1.6%) of the worker-laid egg eating events, and a combination of the queen and 1-2 workers accounted for the three (0.9%) remaining worker-laid egg eating events. Due to the combination of queens and workers eating worker-laid eggs, the number of policing events ($n = 327$) was therefore higher than the number of worker egg-cells eaten ($n = 323$). Queens therefore accounted for 97.3% ($n = 318$) of all observed policing events of worker-laid eggs.

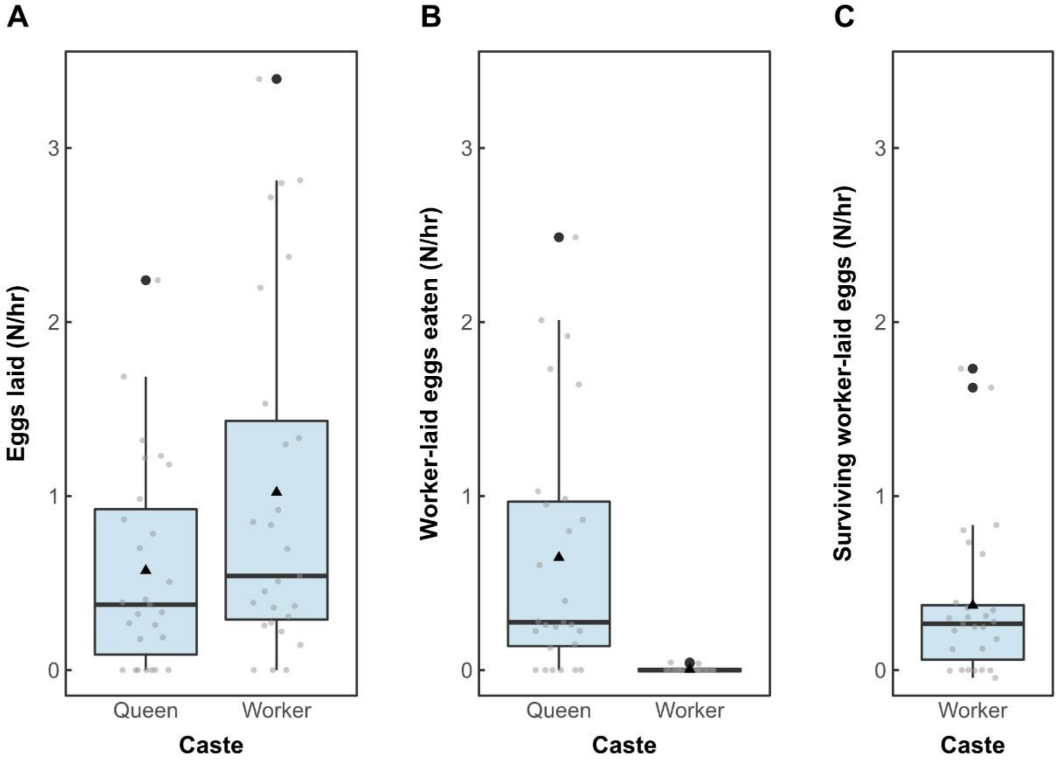

Figure 5.5. Per colony, per hour rates of **(A)** queen and worker egg-laying, **(B)** queen and worker eating of worker-laid eggs; and **(C)** surviving worker-laid eggs across 27 Tree Bumblebee (*Bombus hypnorum*) colonies. Rates are calculated from daily hour-long behavioural recordings under queenright, post-competition point conditions (mean ± SD of 18.7 ± 7.7 days of observational data collected per colony). A total of 320 egg-laying events (n_{queen} = 45, n_{worker} = 275) and 181 egg-eating events (n_{queen} = 179, n_{worker} = 2) were observed across a total of 534.2 hours observed. All egg-eating events observed were the eating of worker-laid eggs. Thick horizontal bars, median; triangles, mean; boxes, interquartile range (IQR); whiskers, range (not including outliers); filled circles, outliers, defined as points more than 1.5 IQR below lower quartile; filled grey circles, 'jittered' raw data, with each point representing a single colony. Note that data came from 27 of the 28 colonies in the present study, since in a single colony (colony 94), the queen died one day after the onset of the competition point. This colony was therefore not used for behavioural observations.

Combining the data from rates of worker-egg laying (with workers accounting for 64.15% of all eggs laid during the competition phase) and survivorship of worker-laid eggs (23.40% survival), workers accounted for 15.01% of eggs remaining two hours post-laying (0.234 × 0.6415). Therefore, assuming no further differential mortality between queen- and worker-laid eggs, the expected contribution of workers to the adult males eclosing from eggs laid during the competition phase was 29.51% (15.01/(35.85 + 15.01)). Worker-produced adult males were found in 11 (52.38%) of the 21 colonies for which adult males were genotyped. During the competition phase, the mean proportion of worker-produced adult males among all genotyped adult males across the 21 colonies was 13.4% ± 21.8%. In fact, frequencies of worker-produced adult males were found to be significantly smaller than those expected from the overall frequencies of worker-egg laying rates and worker-laid egg survivorship (Wilcoxon rank sum test: W = 110.5, $p = 0.015$).

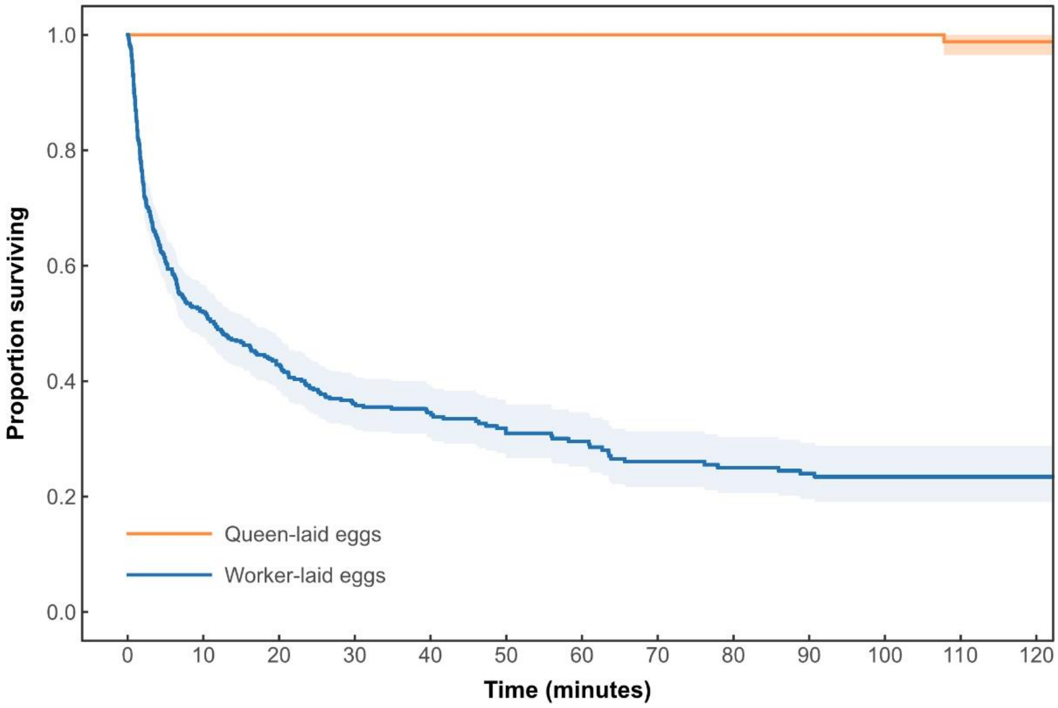

Figure 5.6. Percent survivorship with 95% confidence intervals of queen-laid (orange line; $n = 119$) and worker-laid (blue line; $n = 474$) eggs for up to two hours after being laid in 27 queenright, competition phase Tree Bumblebee (*Bombus hypnorum*) colonies. The single queen-laid egg-eating event occurring at 108 minutes represents an observation of a worker eating two eggs from an egg-cell containing six queen-laid eggs. Note that data came from 27 of the 28 colonies in the present study, since in a single colony (colony 94), the queen died one day after the onset of the competition point. This colony was therefore not used for behavioural observations.

Across all colonies, only 14 individual workers were observed to engage in interrupting laying workers, and only 10 individual workers were observed engaging in egg-eating. Hence, attacking and egg-eating workers were very rare, accounting for 0.004-0.005% of all workers. Egg-laying workers were significantly more likely to attack and interrupt other egg-laying individuals than non-egg layers (Fisher's exact test: $p < 0.001$), with 13 of the 14 (92.86%) attacking workers being egg-layers themselves. Further, egg-eating workers were also significantly more likely to be egg-laying workers than non-egg eating workers were (Fisher's exact test: $p < 0.001$), with 5 of the 10 (50.00%) egg-eating workers being egg-layers themselves.

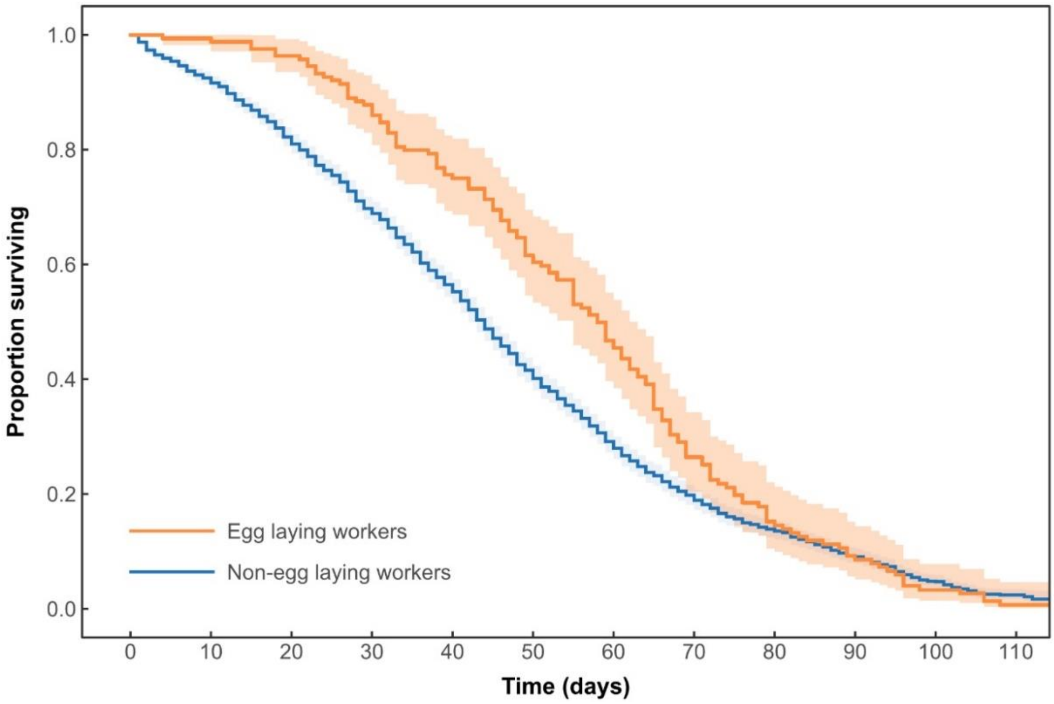

Figure 5.7. Percent survivorship with 95% confidence intervals of egg-laying (orange line; *n* = 164) and non-egg laying (blue line; *n* = 2064) workers, i.e proportion alive as a function of time in days since eclosion, across 27 Tree Bumblebee (*Bombus hypnorum*) colonies. Note that data came from 27 of the 28 colonies in the present study, since in a single colony (colony 94), the queen died one day after the onset of the competition point. This colony was therefore not used for behavioural observations.

(c) Worker longevity

Across all 28 colonies, worker lifespans could be calculated for 2272 (84.1%) of all 2701 workers, comprising 87.0% ± 7.5% of the total workers per colony. The remaining workers were not recovered until the end of the colony rearing period, and so the date of death was unknown (*n* = 429; 15.9%). *B. hypnorum* mean worker longevity was 46.2 ± 25.9 days.

Worker lifespans were available for 164 of the 203 (80.8%) egg-laying workers and for 2063 of the 2648 (77.9%) non-egg-laying workers, identified from the 27 colonies for which competition phase behavioural observation data were available. Egg-laying workers were found to live significantly longer than non-egg laying workers (Log rank test: $\chi^2 = 9.3$, df = 1, $p = 0.002$), with median lifespans of 58 and 44 days, respectively (**Figure 5.7**).

(d) Queen mating frequency and worker-worker relatedness

Across the 420 workers and 19 microsatellite loci, the median number (range) of alleles per locus was 4.0 (2-8), allelic richness was 4.26 ± 1.70, and observed and expected heterozygosity were 0.51 ± 0.20 and 0.52 ± 0.19, respectively (**Table 5.1**).

Paternity reconstruction revealed that 24 of the 28 queens were monandrous, three were doubly mated, and a single queen was triply mated, leading to a population-level numeric mating frequency of 1.18 ± 0.48. All four polyandrous queens were those from the 2019 cohort, and polyandry was therefore detected in 0% of the 2018 queen cohort (*n* = 8) and 20% of the 2019 queen cohort (*n* = 20). In all four cases of queen polyandry, a single male usually dominated worker paternity, with the dominant male siring a 66.67% (range: 53.33-86.70%) share of offspring. Accounting for paternity skew within each colony (Nielsen *et al.*, 2003), the population-level effective queen mating frequency was 1.14 ± 0.39. Intracolony worker-worker relatedness was 0.70 ± 0.11 (range: 0.36-0.83).

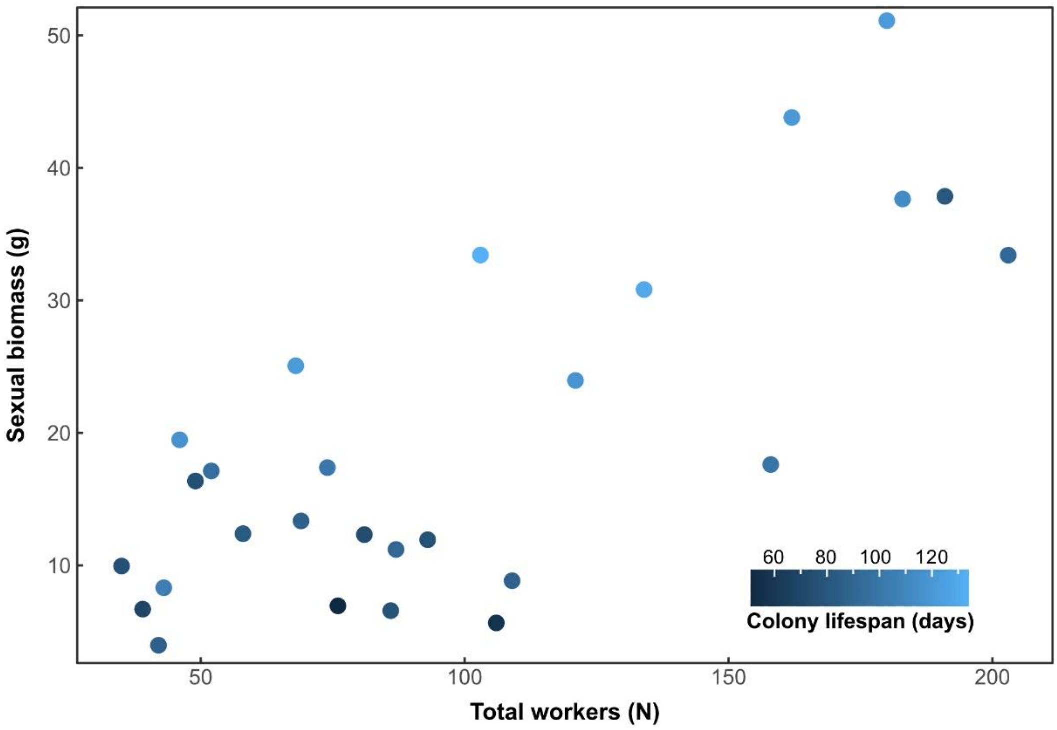

Figure 5.8. Sexual productivity (sexual biomass, i.e. total biomass of gynes and males) of 28 Tree Bumblebee (*Bombus hypnorum*) colonies as a function of the total number of workers produced by a colony and colony lifespan (defined as the time in days between the eclosion of the first worker and the eclosion of the last sexual offspring for each colony). Each point represents a single colony.

Male non-detection error was calculated from allele frequencies (**Table 5.1**) as <0.001 (i.e. less than a 0.1% chance of a second patriline going undetected due to identical father genotypes), suggesting very high resolving powers of the markers. The genotyping of 15 workers from each colony ensured a 95% chance of detecting second patrilines if they sired ≥18% of offspring (Boomsma & Ratnieks, 1996), and so there was a minor chance of male non-sampling error. However, if worker sample sizes were increased and this led to the recovery of additional patrilines, queen effective mating frequencies would still be <2. For instance, if the sample size was increased to 16 and a single, initially non-sampled worker was from a second patriline, the queen effective mating frequency would only be 1.14 (Boomsma & Ratnieks, 1996). Hence, potential error introduced by non-sampling of patrilines is minor for this study.

Table 5.2. Model summary for the effect of the number of workers and the colony longevity on Tree Bumblebee (*Bombus hypnorum*) sexual productivity (total biomass of gynes and males in grams). Parameter estimates and hypothesis tests of all effects included in the 'best' generalised linear model selected using AIC_c (Hurvich & Tsai, 1993; Barton, 2020). Term, name of specified fixed predictor; Estimate, parameter estimate for each predictor; SE, standard error of estimate; *t*, t-test statistic; *p*, p-value, with a value of <0.05 indicating that the null hypothesis of the predictor effect being zero can be rejected.

Term	**Estimate**	**SE**	***t***	***p***
Intercept	0.575	0.311	1.846	0.077
Total workers	0.008	0.001	7.918	<0.001
Colony longevity	0.015	0.003	6.143	<0.001
				Adjusted R^2 = 0.838

Total workers = the total number of workers produced by each colony. *Colony longevity* = the time in days between the eclosion of the first worker and the eclosion of the last sexual offspring (male or gyne) for each colony.

(e) Relationships between productivity, conflict, and polyandry

There was a significant positive relationship between the total sexual productivity of a colony and the total number of workers ($t = 7.918$, $p < 0.001$) and the colony lifespan ($t = 6.143$, $p < 0.001$; **Figure 5.8**; **Table 5.2**). These two predictors were additive, such that colonies that contained a larger number of workers and were longer-lived were more productive (**Figure 5.8**), with the model explaining a high proportion of the variation in sexual productivity (Adjusted $R^2 = 0.838$; **Table 5.2**). No predictors including rates of conflict (proportion of egg-laying workers and rate of worker egg-laying), conflict resolution (rate of queen policing), polyandry (worker-worker relatedness), or other demographic variables (year, parasite infection and queen lifespan) were included in the 'best' model as selected through

AIC_c comparison (Hurvich & Tsai, 1993; Barton, 2020), suggesting that such terms had non-significant impacts on colony sexual productivity.

A significant positive relationship was also found between colony sex ratio and the total number of workers ($z = 3.003$, $p = 0.003$) and the colony lifespan ($z = 2.746$, $p = 0.006$; **Figure 5.9**; **Table 5.3**). Once again, these two predictors were additive, such that longer-lived colonies containing larger numbers of workers produced a more female-biased sex-ratio (**Figure 5.9**), with the model capturing a moderate proportion of sex-ratio variance (Pseudo $R^2 = 0.438$; **Table 5.3**). As in the productivity model, no predictors including conflict rates, conflict resolution, polyandry, year, parasite infection or queen lifespan were included in the AIC_c selected 'best' model, suggesting that such terms had non-significant impacts on colony sex ratio.

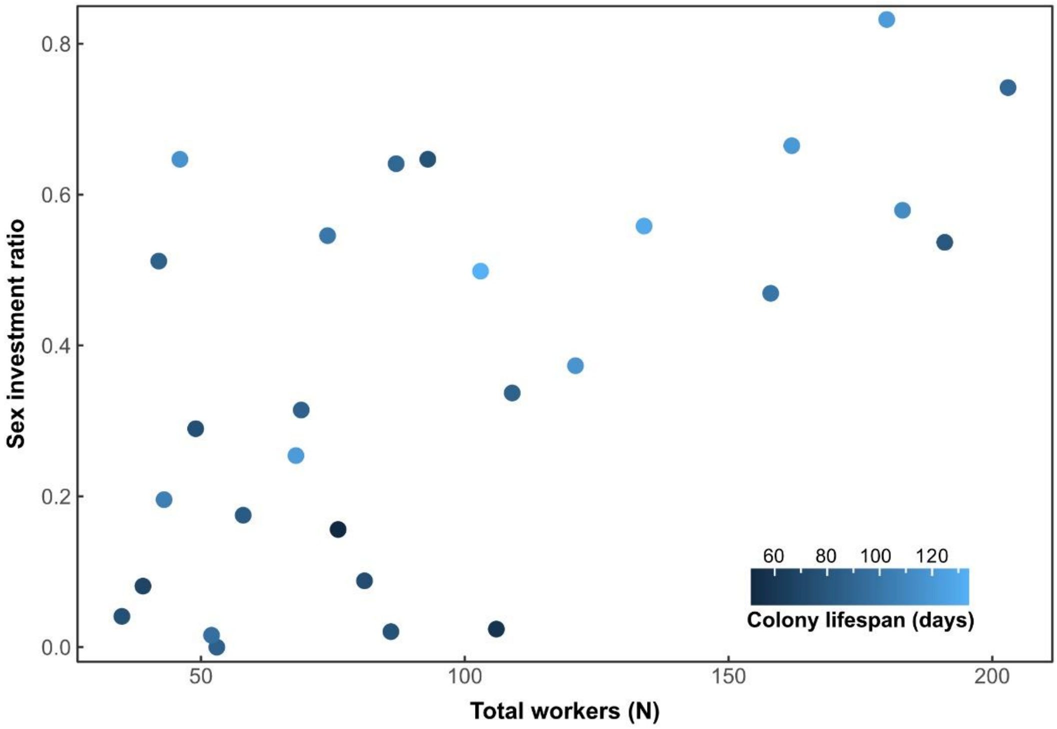

Figure 5.9. Sex investment ratio (calculated as the total gyne biomass divided by total sexual biomass) of 28 Tree Bumblebee (*Bombus hypnorum*) colonies as a function of the total number of workers produced by a colony and colony lifespan (defined as the time in days between the eclosion of the first worker to the eclosion of the last sexual offspring for each colony). Each point represents a single colony.

Table 5.3. Model summary for the effect of the number of workers and the colony longevity on Tree Bumblebee (*Bombus hypnorum*) sex investment ratio (total biomass of gynes divided by total sexual biomass). Parameter estimates and hypothesis tests of all effects included in the 'best' beta regression model selected using AIC$_c$ (Hurvich & Tsai, 1993; Barton, 2020). Term, name of specified fixed predictor; Estimate, parameter estimate for each predictor; SE, standard error of estimate; *z*, Wald z-test statistic; *p*, p-value, with a value of <0.05 indicating that the null hypothesis of the predictor effect being zero can be rejected.

Term	Estimate	SE	*z*	*p*
Intercept	-3.656	0.773	-4.731	<0.001
Total workers	0.010	0.003	3.003	0.003
Colony longevity	0.022	0.008	2.746	0.006
				Pseudo R^2 = 0.438

Total workers = the total number of workers produced by each colony.
Colony longevity = the time in days between the eclosion of the first worker and the eclosion of the last sexual offspring (male or gyne) for each colony.

5.5. Discussion

This study represents the first quantitative analysis of the behavioural processes and subsequent outcomes (realised proportions of worker-produced adult males) of male production conflict in Tree Bumblebee (*Bombus hypnorum*) colonies. Further, it also represents the second such study across all bumblebee species, with comparable data only available for the well-studied *B. terrestris* (Zanette *et al.*, 2012), and one of few studies across annual eusocial Hymenoptera as a whole (Foster & Ratnieks, 2000). Specifically, our findings show that in *B. hypnorum*: (1) workers lay the majority of eggs laid following the competition point, but low survivorship of worker-laid eggs due to policing leads to few worker-produced adult males;

and (2) queens carried out almost all of the observed policing, with workers very rarely partaking in policing behaviour. Further, I found no links between conflict resolution, polyandry, and colony productivity in *B. hypnorum*, although this was likely due to the scarcity of worker policing and low effective mating frequency in the study *B. hypnorum* population. Hence, conflict resolution in *B. hypnorum* more closely resembles those mechanisms observed in small eusocial insect societies, where control over reproduction stems mainly from queen control (e.g. Bourke, 1991; Nakata & Tsuji, 1996; Monnin & Peeters, 1997).

(a) Conflict resolution in *B. hypnorum*

As predicted by kin selection theory (Hamilton, 1964b; Trivers & Hare, 1976; Queller & Strassmann, 1998; **Figure 5.1**), actual conflict between the queen and her workers over male production in *B. hypnorum* colonies was extremely high. Worker egg-laying was observed in 92.59% of all colonies, with egg-laying workers constituting 7.6% of workers and workers collectively producing ~64% of all eggs laid during the competition phase (**Figure 5.5**). However, successful worker reproduction was limited through two specific policing mechanisms. Firstly, the number of eggs that workers were able to lay was limited pre-emptively through aggression and subsequent interruption of the egg-laying worker during oviposition, leading to significantly fewer eggs being laid (**Figure 5.4B**). Queens carried out the majority of such interruptions, accounting for 143 (84.12%) of the 170 observed egg-laying interruptions, and in 27 cases, interruption meant the ovipositing worker failed to lay a single egg (**Figure 5.4B**), with 25 (92.59%) of the failed attempts attributable to queen attack.

Secondly, if workers managed to successfully lay eggs, survival of worker-laid eggs was low due to selective eating of worker-laid eggs (23.4% chance of survival two hours post-laying; **Figure 5.6**). Once again, queens carried out most worker-laid egg eating, accounting for 318 (97.25%) of the 327 observed events. Such policing led to relatively low proportions of worker-produced adult males, with worker-produced adult males being found in

52.38% of all colonies, with 13.4% of adult males that eclosed from eggs laid during the competition phase found to be worker-produced. These findings are congruent with those of Brown *et al.* (2003), which found workers accounted for 19.6% of adult males. Interestingly, the observed frequencies of worker-produced males were significantly lower than those predicted from the overall frequencies of worker-egg laying rates and worker-laid egg survivorship. One potential reason for this finding is that worker-laid eggs may continue to be eaten beyond two hours post-laying. For instance, in the wasps *Dolichovespula sylvestris* and *D. norwegica* worker-laid eggs continue to be eaten for up to 16 hours after being laid (Wenseleers *et al.*, 2005b; Bonckaert *et al.*, 2011a).

Given kin-selected predictions regarding male production conflict in the eusocial Hymenoptera (**Figure 5.1**), the existence of queen policing in *B. hypnorum* is unsurprising, and corroborates a previous study of a single *B. hypnorum* colony that found the queen detected and ate all worker-laid eggs (Bollingmo, 1989). Interestingly, queen policing rates in *B. hypnorum* are far higher than those for a number of annual eusocial species with comparable social organisation; 97.25% of policing events are carried out by the queen in *B. hypnorum* vs. 32% in *D. norwegica* (Bonckaert *et al.*, 2011a), 49% in *D. sylvestris* and *B. terrestris* (Wenseleers *et al.*, 2005b; Zanette *et al.*, 2012), and 58% in *Vespula rufa* (Wenseleers *et al.*, 2005a). Patterns of queen policing in *B. hypnorum* therefore fit with previous predictions that queen control over reproductive conflict can evolve in eusocial species with relatively small colony sizes (Ratnieks, 1988; Bourke, 1999), and may explain the lack of worker-produced adult males observed in Paxton *et al.* (2001).

There are a number of factors that may select for worker policing, including relatedness differences driven by polyandry (Ratnieks, 1988; Crozier & Pamilo, 1996), sex-ratio biasing (Foster & Ratnieks, 2001c), increased colony productivity (Hartmann *et al.*, 2003; Teseo *et al.*, 2013), or increased reproductive success of the policing worker (‘selfish policing’; Stroeymeyt *et al.*, 2007; Bonckaert *et al.*, 2011; Zanette *et al.*, 2012). Despite this, and unlike the closely related *B. terrestris*, worker policing was extremely rare across colonies of *B. hypnorum* (**Figure 5.5B**), with workers only accounting for 5.22% and 2.75% of attacks on ovipositing workers and worker-laid egg eating events, respectively. Further, workers that engaged in policing behaviour constituted just 0.004-0.005% of all workers. Interestingly, the few workers that engaged in policing were significantly more likely to be reproductive themselves, suggesting that selfish policing may explain worker policing in *B. hypnorum*, matching patterns in other annual eusocial species (Bonckaert *et al.*, 2011a; Zanette *et al.*, 2012).

Ultimately, the rarity of worker policing in *B. hypnorum* may be explained by the effectiveness of queen policing, in that workers do not need to expend energy policing other workers' eggs given the high likelihood of policing being carried out by the queen. Alternatively, workers may refrain from policing based on kin-selected grounds, since under monandrous conditions, or conditions approximating monandry, workers are more highly related to their nephews ($r = 0.375$) than their brothers ($r = 0.25$; **Figure 5.1**). Indeed, that worker policing occurs in *B. terrestris* (subgenus: *Bombus*) but not in *B. hypnorum* (subgenus: *Pyrobombus*) suggests that worker policing may be an evolutionary derived state in the *Bombus* subgenus, rather than having evolved in the common ancestor of the two subgenera. Future studies could combine a phylogenetic approach with the collection of further conflict resolution data across a range of bumblebee subgenera to provide insight into the evolution of worker policing across bumblebees.

In contrast to the high interruption rates of egg-laying workers and high rates of eating of worker-laid eggs, policing behaviour was very rarely directed towards the queen in the present study. Queen egg-laying was only interrupted by worker attack in 3 (2.38%) of the 126 observed queen laying events, while 99.6% of all queen eggs laid during the competition phase survived for up to two hours (**Figure 5.6**). Differential survivorship between queen- and worker-laid eggs is a hallmark of worker policing across multiple eusocial species (Ratnieks & Visscher, 1989; Foster & Ratnieks, 2001b;

Halling *et al.*, 2001; Wenseleers *et al.*, 2005b; Bonckaert *et al.*, 2011a; Zanette *et al.*, 2012). At the proximate level, one possible reason for workers avoiding eating queen-laid eggs may be due to different chemical cues present on queen- and worker-laid eggs, with workers able to distinguish between such cues and actively avoid policing queen eggs (Zanette *et al.*, 2012; Oi *et al.*, 2020a). Alternatively, given that *B. hypnorum* queens continue laying diploid eggs after switching to haploid egg production (**Chapter 4**), workers may avoid eating queen-laid eggs since they risk eating highly valuable sisters (**Figure 5.1**). However, it is difficult to say whether *B. hypnorum* workers actively avoid policing queen-laid eggs, or whether the high survivorship of queen-laid eggs is a by-product of worker egg-eating being rare overall.

The outcomes of male production conflict in *B. hypnorum* are similar to those observed in other annual eusocial Hymenoptera species. For example, workers collectively account for 69% and 50% of eggs laid in *B. terrestris* and *D. sylvestris* colonies respectively, but worker-laid egg survival rates of 6-9% lead to realised worker-produced male proportions of ~10% in both species (Foster *et al.*, 2001; Wenseleers *et al.*, 2005b; Zanette *et al.*, 2012). However, in contrast to the present study, where eating of worker-laid eggs is carried out almost exclusively by the queen, eating of worker-laid eggs in both *B. terrestris* and *D. sylvestris* is split roughly 50:50 between the queen and her workers (Wenseleers *et al.*, 2005b; Zanette *et al.*, 2012). Hence, the present study demonstrates that there are multiple routes to effective reproductive conflict resolution in annual eusocial insect societies, ranging from complete queen policing (as in *B. hypnorum*), to both queen and worker policing (as in *B. terrestris* and *D. sylvestris*; Wenseleers *et al.*, 2005b; Zanette *et al.*, 2012), to complete worker policing (as in *V. vulgaris*; Foster & Ratnieks, 2001b). Further collection of conflict resolution data across a broad range of eusocial Hymenoptera would allow investigation into the phylogenetic relationships between these different mechanisms of conflict resolution, providing insight into the origin and elaboration of worker policing behaviour.

(b) Longevity of egg-laying workers

An additional finding from this study was that egg-laying *B. hypnorum* workers were significantly longer-lived than non-egg laying workers (**Figure 5.7**), matching previous findings in both *B. terrestris* and *Apis mellifera* (Blacher *et al.*, 2017; Kuszewska *et al.*, 2017) and more broadly matching the reversal of negative fecundity-longevity relationships exhibited by social insect queens as a whole (Kramer *et al.*, 2016). In *B. terrestris*, such a relationship was reversed (i.e. the costs of reproduction were incurred) when workers were forced into becoming reproductive, suggesting that positive fecundity-longevity relationships are the result of higher quality workers that are able to overcome the costs of reproduction (Blacher *et al.*, 2017). Conceivably, such patterns between worker quality, reproduction and longevity are also present in *B. hypnorum*, although experiments like those of Blacher *et al.* (2017) would be required to definitively prove this. One potential caveat to this result is that some workers may have been misclassified as non-egg-laying if they laid eggs outside the observation periods, since ovarian dissections were not carried out on the workers. However, given the current effect size of egg-laying on worker-longevity (**Figure 5.7**) there is little reason to think that misclassification of a worker reproductive status presents a major issue for this finding.

(c) Relationships between productivity, conflict, and polyandry

In agreement with previous studies (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000; Paxton *et al.*, 2001; Brown *et al.*, 2002, 2003; Crowther *et al.*, 2019), I found evidence for facultative polyandry in the studied *B. hypnorum* population(s), with numeric and effective queen mating frequencies of 1.18 and 1.14, respectively. Interestingly, queen mating frequency was lower than that found in the same UK population from Crowther *et al.* (2019), who found a numeric mating frequency of 1.7, with the majority of queens being doubly-mated. Differences between the two studies may be the result of different methodologies for estimating the level of polyandry. Crowther *et al.* (2019) estimated mating frequency through genotyping of the sperm inside the spermatheca, whereas the present study did so by genotyping adult workers within colonies. Accordingly, the UK *B. hypnorum* population may exhibit high levels of paternity skew, as are found in other eusocial Hymenoptera (Boomsma & Ratnieks, 1996). Alternatively, queen mating frequency may be an annually variable trait, with differences in mating frequency between years, as observed in the obligately polyandrous wasp *V. maculifrons* (Dyson *et al.*, 2021). For instance, in the present study, no queens from the 2018 cohort were polyandrous, but 20% of queens from the 2019 cohort exhibited polyandry, although this difference was non-significant (Fisher's exact test: p = 0.29).

Finally, no evidence was found for the impact of reproductive conflict, conflict resolution or queen mating frequency on measures of colony productivity (**Table 5.2; Table 5.3**). Such findings were potentially influenced by the limited predictive power of comparing four polyandrous colonies to 23 monandrous colonies, along with the distinct lack of worker policing observed in the *B. hypnorum* colonies. Alternatively, the costs of worker reproduction may accrue non-linearly in *B. hypnorum* colonies (Foster, 2004; Archetti *et al.*, 2020), such that colonies are capable of supporting some level of worker reproduction without any apparent cost to colony productivity. Indeed, such a relationship has previously been observed in *B. terrestris* colonies, in which levels of worker reproduction had no discernible negative effect on the total number of gynes produced (Lopez-Vaamonde *et al.*, 2003). Regardless, due to low effective mating frequencies and the scarcity of worker policing behaviour, the UK *B. hypnorum* population is not suited to testing inclusive fitness-based predictions between polyandry and worker policing (Wenseleers & Ratnieks, 2006) or polyandry and split-sex ratios (i.e. where colony sex investment ratios vary depending on worker-worker relatedness; e.g. Boomsma & Grafen, 1991; Chan & Bourke, 1994; Meunier *et al.*, 2008). Instead, the best measures to explain colony productivity and sex ratio were the number of workers a colony contained and the colony lifespan, such that longer-lived colonies with high numbers of workers were both more sexually productive (**Figure 5.8**; **Table 5.2**) and produced more female-biased sex ratios (**Figure 5.9**; **Table 5.3**), similar to previous findings in *B. hypnorum* (**Chapter 4**) and across other eusocial species (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015).

(d) Conclusions

Overall, this study provides valuable insight into queen-worker conflict over male production in the Tree Bumblebee (*B. hypnorum*), and the behavioural processes that help resolve it. Conflict resolution mechanisms in *B. hypnorum* show distinct similarities and differences relative to other annual eusocial species for which comparable data are available. Similarities include the policing of worker reproduction through both direct attack of ovipositing workers and through the targeted eating of worker-laid eggs (**Figure 5.2C-D**; **Figure 5.6**). However, conflict resolution in *B. hypnorum* is distinct in that such behaviours are carried out almost exclusively by the queen, as opposed to by both the queen and her workers (e.g. Wenseleers *et al.*, 2005b; Zanette *et al.*, 2012) or exclusively by the workers (Foster & Ratnieks, 2001b). Impressively, centralised queen policing alone largely reduces realised worker reproduction in *B. hypnorum*, though such an outcome was previously considered unlikely (Hammond & Keller, 2004). These findings highlight the diversity of conflict resolution mechanisms across eusocial insect societies, demonstrate that there are multiple routes through which selfish interests dissolutive to social group maintenance can be managed in eusocial evolution, and raise new questions about the evolutionary pathways between different modes of conflict resolution.

5.6. References

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Chapter 6

General conclusions

A Tree Bumblebee (*Bombus hypnorum*) worker foraging on bramble (*Rubus fruticosus*).

CHAPTER 6

General conclusions

6.1. Thesis summary

The colonisation and ecological success of the Tree Bumblebee (*Bombus hynorum*) in the UK represents an illuminating case study for the investigation of the population genetic processes associated with successful range-expansions and the life-history traits associated with ecological success. Further, the occurrence of facultative polyandry in *B. hypnorum* queens in the UK (Crowther *et al.*, 2019) provides an opportunity to empirically test inclusive fitness predictions regarding the resolution of reproductive conflict within eusocial societies. Accordingly, using a combination of genetic, demographic, and behavioural approaches, this thesis aimed to address specific elements relating to the population genetics and ecological success of *B. hypnorum* in the UK, and the resolution of male production conflict in eusocial societies. Specific techniques included microsatellite genotyping, developing and refining means for captive-rearing of mature colonies from field-collected *B. hypnorum* queens, long-term captive colony censusing and monitoring, assessment of parasite load via dissection and phase-contrast microscopy, and behavioural analysis of the within-nest behaviour of individually-marked bees via direct observation and digital filming.

Briefly, the key findings of this thesis are as follows. In **Chapter 2**, I found that *B. hypnorum* did not undergo a genetic bottleneck upon colonising the UK, as it shows levels of genetic diversity intermediate between those of widespread and rare *Bombus* species and relatively low frequencies of diploid male production (Brock *et al.*, 2021). In **Chapter 3**, I found that *B. hypnorum* demonstrates partial resistance to the castrating effects of the nematode parasite, *Sphaerularia bombi*, and that *B. hypnorum* queens may represent a dead-end host for the parasite, with *S. bombi* rarely managing to successfully reproduce within their *B. hypnorum* host. In **Chapter 4**, I characterised the colony demography of *B. hypnorum*, finding evidence to suggest that *B. hypnorum* exhibits higher levels of gyne production than other native UK bumblebee species that, unlike the range-expanding *B. hypnorum*, exhibit stable population trends. Further, I demonstrated positive relationships between queen-lifetime reproductive success and various individual- and colony-level life-history traits, including queen longevity, colony size, and colony longevity. Finally, in **Chapter 5**, I demonstrated that queen-worker conflict over male production is resolved almost exclusively by queen policing, with such policing effectively limiting the direct fitness gains of selfish reproductive workers.

The rest of this chapter aims to briefly synthesise the findings from each chapter, discuss their implications, and suggest future directions for further research.

(a) Population genetics of a range-expanding bumblebee

Previous research suggested that *B. hypnorum* experienced a severe genetic bottleneck upon colonising the UK, with a founding population of as few as one or two polyandrous queens (Jones & Brown, 2014). Accordingly, the successful colonisation and ecological success of *B. hypnorum* in the UK has previously been cited as a prime example of the genetic paradox of invasion (Schrieber & Lachmuth, 2017). However, analysis of genotypic data from *B. hypnorum* workers and males in **Chapter 2** showed relatively high allelic diversity at neutral microsatellite loci and (based on relatively low levels of diploid male production) at the sex-determining locus, providing no evidence of a genetic bottleneck coinciding with *B. hypnorum*'s initial colonisation of the UK (Brock *et al.*, 2021). Therefore, these results do not support the suggestion that the UK *B. hypnorum* population suffered a severe genetic bottleneck (Jones & Brown, 2014), nor that *B. hypnorum*'s success in the UK represents an example of the genetic paradox of invasion (Schrieber & Lachmuth, 2017). Instead, these results support the idea that colonisation of the UK represents part of *B. hypnorum*'s long-term, westward range expansion across Europe (Wagner, 1937; Rasmont, 1989; Rasmont *et al.*, 2015; Rollin *et al.*, 2020), with immigration continuing to occur between UK and mainland European populations. Therefore, *B. hypnorum* may resemble other invertebrate taxa that are expanding in range at a continental scale, such as the Wasp Spider, *Argiope bruennichi* (Krehenwinkel & Tautz, 2013) and the Dainty Damselfly, *Coenagrion scitulum* (Swaegers *et al.*, 2013).

Future research could test whether *B. hypnorum*'s range expansion has involved a loss of genetic diversity at the leading edge of expansion (Ibrahim *et al.*, 1996) and the associated impacts on adaptability, by sampling multiple *B. hypnorum* populations across its Eurasian range. Research might also focus on the environmental and genetic factors that have led to *B. hypnorum*'s range-expansion and subsequent success. Specifically, studies might assess whether the range-expansion of *B. hypnorum* is associated with climate change, as reported in other bumblebees and insects (e.g. Breed *et al.*, 2013; Martinet *et al.*, 2015), and how a changing climate may alter *B. hypnorum*'s future range and ecology (Martinet *et al.*, 2020; Suzuki-Ohno *et al.*, 2020; Maebe *et al.*, 2021). Finally, future studies might try to unveil the genetic processes that trigger range expansions away from source populations, or the genomic signatures that are associated with *B. hypnorum*'s ecological success (Theodorou *et al.*, 2018; Huml *et al.*, 2021).

(b) Host-parasite interactions in a range-expanding bumblebee

Previous research has shown that the UK *B. hypnorum* population exhibits parasite prevalence and community structure similar to those of other native bumblebee species (Jones & Brown, 2014), suggesting that its ecological success does not represent an example of the enemy release hypothesis (Torchin *et al.*, 2003). However, other parasite-mediated mechanisms may explain the success of *B. hypnorum* as a range-expanding bumblebee.

In **Chapter 3**, I carried out the first quantitative assessment of host-parasite interactions between *B. hypnorum* and *S. bombi*, a castrating nematode parasite (Rutrecht & Brown, 2008; Kelly, 2009) that has anecdotally been reported to impact *B. hypnorum* queens to a lesser extent than in other *Bombus* species (Hasselrot, 1960; Röseler, 2002; Jones & Brown, 2014). As in Jones & Brown (2014), I found that the UK *B. hypnorum* population shares a parasite prevalence and community structure similar to those of native UK bumblebees, providing further evidence that *B. hypnorum*'s success in the UK is not due to escape from parasites. In addition, I showed that *B. hypnorum* queens were partially resistant to the castrating effects of *S. bombi* infection, with infected queens still being able to lay eggs and, more rarely, found colonies and produce sexuals. However, *S. bombi* infection came at a significant cost to queen fitness, with infected queens rearing fewer offspring and exhibiting shorter longevities than uninfected queens. Nonetheless, that a subset of queens can produce sexuals despite *S. bombi* infection may represent one factor underlying *B. hypnorum*'s success in the UK. Finally, while *S. bombi* females were capable of infecting *B. hypnorum* queens and reaching reproductive maturity within the haemocoel, *S. bombi* females were rarely able to reproduce inside the host, with juvenile stages never being found in the host faeces or hindgut and rarely being found in the host haemocoel. These findings suggest that *S. bombi* cannot successfully complete its life-cycle upon infecting *B. hypnorum* (i.e. *B. hypnorum* represents a dead-end host for *S. bombi*), with potential benefits for other native bumblebee species through parasite dilution effects.

Future research could analyse the transcriptomic changes associated with *S. bombi* infection and compare these to those observed in *B. terrestris* (Colgan *et al.*, 2020), potentially providing insight into the physiological and immunological mechanisms that lead to *B. hypnorum*'s resistance to *S. bombi*. Given that such studies would require experimental infection of hibernating *B. hypnorum* queens, methods that allow the rearing of *B. hypnorum* through its complete life-cycle in captive conditions (i.e. hibernation, colony founding, sexual production, and mating; **Figure 1.1**) would need to be developed. Such methods would be challenging to develop, given that a large proportion of queens would be lost at each stage (e.g. the highest success rate of captive colony foundation from field-collected queens in **Chapter 4** was 22%). Hence, collaborations with large-scale, commercial bumblebee rearing companies, such as BioBest, may be required to perfect such techniques in *B. hypnorum* (Velthuis & Van Doorn, 2006). Further research might also focus on how *B. hypnorum*'s dead-end host status impacts the prevalence of *S. bombi* in other native bumblebee species; for example, if such an effect occurs, it would predict that *S. bombi* prevalence decreases in other bumblebee species when *B. hypnorum* is abundant at a given location.

(c) Colony demography and queen lifetime fitness in a range-expanding bumblebee

Despite growing interest in the fecundity-longevity trade-off in social insects (Korb & Heinze, 2021), the relationship between queen longevity and reproductive success is understudied in the eusocial Hymenoptera (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015). This is more broadly reflective of the scarcity of quantitative data on colony-level life-history traits across the eusocial Hymenoptera, despite the potential insight into social evolution provided by such data (Tschinkel, 1991; Cole, 2009).

In **Chapter 4**, I provided the first comprehensive characterisation of colony demography in the range-expanding bumblebee *B. hypnorum*, allowing comparisons with the well-studied *B. terrestris* to illuminate potential demographic factors that might explain *B. hypnorum*'s ecological success in the UK. I found that *B. hypnorum* shows colony growth patterns similar to those of *B. terrestris*, but lacked as clear-cut a switch-point, with queens continuing to lay both haploid and diploid eggs during the reproductive phase of the colony cycle. Interestingly, *B. hypnorum* colonies appeared to be significantly more gyne-productive than *B. terrestris* and, given that queens represent the dispersing and founding propagule in bumblebees, this higher gyne-productivity may be one factor explaining *B. hypnorum*'s rapid population growth and range-expansion across the UK (**Figure 1.2**). Finally, I found that queen-lifetime reproductive success is positively associated with queen longevity but is also influenced by other individual- and colony-level traits, such as latency to colony foundation, colony size and colony longevity. Alongside previous studies in both annual and perennial eusocial species (Lopez-Vaamonde *et al.*, 2009; Heinze *et al.*, 2013; Kramer *et al.*, 2015), these findings suggest that positive relationships between queen longevity and fitness are universal across the eusocial Hymenoptera, conceivably stemming from prolonged parent-offspring interactions in their solitary ancestors. Future research should continue to collect life-history data across the spectrum of social diversity within the eusocial Hymenoptera, providing further insight into the relationship between sociality, ageing and longevity.

(d) Reproductive conflict resolution in a eusocial society

Conflict between the queen and her workers over male-production is widespread in colonies of eusocial Hymenoptera since, under haplodiploidy, workers are able to lay haploid, male-destined eggs to which they are more highly related than they are to queen-produced males (Bourke, 1988; Hammond & Keller, 2004; **Figure 5.1**). Such conflict represents a dissolutive force during social evolution (Bourke, 2011) but can be resolved through policing, a coercive mechanism in which queens and/or workers limit the reproductive success of workers via aggression towards reproductive workers and the destruction of worker-laid eggs (Ratnieks, 1988; Wenseleers & Ratnieks, 2006).

In **Chapter 5**, I aimed to use the facultatively polyandrous nature of *B. hypnorum* (e.g. Estoup *et al.*, 1995; Paxton *et al.*, 2001; Almond *et al.*, 2019) to test inclusive fitness-based predictions of worker policing, specifically that worker policing should evolve under polyandry and/or as a mechanism of increasing colony productivity (Ratnieks, 1988; Hartmann *et al.*, 2003). Contrary to expectations, I found that worker policing was almost entirely absent in *B. hypnorum*, with the reproductive success of workers being effectively limited by two distinct queen-policing mechanisms: aggression towards egg-laying workers and eating of worker-laid eggs. Further, neither rates of conflict, rates of policing, nor queen polyandry were significant predictors of colony productivity. However, this might have been due to reduced statistical power from only four of the queens being multiply-mated. Despite a lack of worker policing and low levels of queen polyandry preventing tests of the original hypotheses, **Chapter 5** represents the first quantitative study of conflict resolution in *B. hypnorum* (and one of few across the eusocial Hymenoptera as a whole) and highlights queen policing as an important mechanism of conflict suppression in eusocial Hymenoptera and as a potential precursor to the evolution of other mechanisms including policing by both reproductive and non-reproductive workers. Such findings also demonstrate the diversity of conflict resolution strategies found across eusocial insect societies.

Given that my research shows worker policing is unlikely to occur regardless of queen mating frequency, future research in the system might investigate alternative benefits of polyandry in *B. hypnorum*. For instance, does polyandry confer greater parasite resistance (e.g. Saga *et al.*, 2020), reduce the risk of worker matricide (e.g. Loope, 2015), or increase general adaptability and ecological success (e.g. Ding *et al.* 2017; Lewis *et al.*, 2020) in *B. hypnorum*? Manipulation of queen mating frequency under controlled conditions may also lead to an increased understanding of sexual selection in eusocial insects (Boomsma & Ratnieks, 1996; Baer, 2003; den Boer *et al.*, 2010); for example, under polyandrous conditions, what male traits lead to an increased paternity share among the queens offspring?

6.2. Concluding remarks

Taken together, the findings from each chapter presented in this thesis make key contributions to our understanding of the genetic and life-history features underlying ecological success in a range-expanding bumblebee, as well as mechanisms regulating kin-selected conflict in eusocial societies. In particular, the UK *B. hypnorum* population exhibits relatively high levels of genetic diversity and no evidence of a severe genetic bottleneck (Brock *et al.*, 2021), and hence is not expected to suffer from the negative fitness effects associated with low genetic diversity. Further, *B. hypnorum* appears to show some resistance to the castrating effects of *S. bombi* observed in native bumblebee species, and increased levels of gyne investment when compared to the abundant *B. terrestris*. Hence, alongside its aerial nesting behaviour (Prŷs-Jones, 2014, 2019) and distinct habitat and foraging niche (Crowther *et al.*, 2014), *B. hypnorum* appears to possess multiple traits that make it unique among the British bumblebee fauna, and may explain its rapid range-expansion and ecological success in the UK. Finally, reproductive conflict is widespread in *B. hypnorum* colonies, but the reproductive success of workers is successfully limited via queens aggressing ovipositing workers and eating their eggs. Hence, conflict resolution strategies in *B. hypnorum* resemble those in small eusocial societies, where control over reproduction stems mainly from queen control.

Overall, this thesis makes a significant contribution to three broad areas of wide interest in the general field of evolution and ecology, namely (1) the biological basis of range expansions and invasiveness; (2) the ecology, colony demography, parasitology, genetics, and conservation of bumblebees as a key group of pollinating insects; and (3) the evolution of social behaviour and particularly the maintenance of social groups through conflict resolution.

6.3. References

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