The cryptic life cycle stages of bumble bees: using natural history to guide conservation.

A dissertation submitted by:

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In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Biology

TUFTS UNIVERSITY

August 2021

ADVISOR: Dr. Elizabeth E. Crone

Summary abstract

Bumble bees (*Bombus* spp.) are a taxon of great importance to agricultural systems and natural communities. Rapid declines of bumble bee populations in North America and Europe have generated considerable interest in improving and protecting remaining habitat. However, the methodologies commonly used to investigate bumble bee habitat rarely inform our understanding of resource requirements during cryptic life cycle stages. My dissertation research, in contrast, addresses crucial knowledge gaps related to these life cycle stages, in addition to pointing out some of the strengths and limitations of common methods used to study bumble bees.

Chapters 2 and 3 of my dissertation target bumble bee nest founding, which is likely one of the most sensitive stages of the life cycle. In Chapter 2, I explore the nesting ecology of bumble bees at multiple spatial scales, and utilize modern statistical tools to estimate bumble bee nest densities across different land cover types. I found that North American bumble bees exhibit species-specific patterns of nesting habitat use: for example, *B. impatiens* nested in subterranean nest sites in both forests and in open grasslands, whereas *B. griseocollis* typically nested on the surface of the ground, almost exclusively in meadows. This chapters also highlights a previously overlooked feature of bumble bee ecology: that nesting habitat use and habitat quality are not perfectly aligned. Although *B. impatiens* nests were found at similar densities in both forests and meadows, colonies nesting in forests were more successful in terms of reproductive output compared those nesting in meadows. In Chapter 3, I demonstrate movement of nest-searching queens (*B. impatiens*) as a promising metric for identifying bumble nesting habitat. To quantify movement, I followed queen *B. impatiens* searching for nests in different land cover types, and related patterns observed to estimates of nest density (presented in Chapter 2). I found that nest-

searching queens moved in a way that would decrease their rate of movement in land cover types associated with high nest densities (forests and meadows) than in non-habitat (hayfields). Together, these chapters provide valuable insights an incredibly understudied stage of the bumble bee life cycle, and showcase the benefits and challenges of studying bumble bee nesting ecology in the field.

The next two chapters of my dissertation address the limitations of two common methods of studying bumble bees. In Chapter 4, I explore whether commercial bumble bees, which are commonly used as scientific subjects in ecological research, are representative of bumble bees in nature. By collecting workers at nest entrances, I compared behavioral and morphological characteristics of wild and commercial workers. I found that, although commercial and wild bumble bees foraged on similar communities of flowers, wild bumble bees were larger, returned to colonies with purer pollen baskets, and were more likely to return to the colony with pollen than their commercial counterparts. In Chapter 5, I explored whether the diapause mortality rates of queen bumble bees estimated by lab-based studies are comparable to mortality rates of natural bumble bee populations. To estimate diapause survival for bumble bees in the wild, I monitored four aggregations of overwintering *B. impatiens* queens. I also conducted a meta-analysis of past literature, in order to draw comparisons between my field-based estimates of survival and those produced by lab-based studies, finding that queens overwintering in the field had far higher rates of overwintering survival. Together, these studies emphasize that researchers studying bumble bees in the lab, or using commercial bees as study subjects, ought to exercise caution in generalizing their findings to wild bee populations, and highlight the value of pairing controlled, lab-based experiments with observations of wild bumble bees.

Acknowledgements

Words cannot express my gratitude to my colleagues, friends, and loved ones for their support in this journey.

First and foremost, I would like to thank my advisor, Dr. Elizabeth Crone, for welcoming me into her lab group. I am so, so glad that I chose to attend graduate school at Tufts. I could not have asked for a better mentor to guide me through the most formative years of my career. Thank you, Elizabeth, for always believing in me. You are truly an amazing mentor, and it has been an incredible privilege to work with you.

There are many other folks within the Tufts community who deserve special acknowledgement for their role in my professional development. I would like to thank the other members of my committee, Dr. Colin Orians, Dr. Michael Reed, and Dr. Philip Starks, not only for their mentorship, but for granting me additional teaching opportunities while I was a graduate student at Tufts. Dr. Starks, especially, shaped much of my training as a TA. I would also like to acknowledge Dr. David Iles, who, during his short time at Tufts, inspired much of my dissertation work.

Of course, I must also thank my wonderful lab mates, both past and present: Sylvie Flynn, James Michielini, Dr. Collin Edwards, Atticus Murphy, Dr. David Iles, Dr. Natalie Kerr, Nick Dorian Dr. Lee Brown, and Dr. Norah Warcola, who inspired me in so many ways. I am so grateful I have had the opportunity to work with some of the most dedicated and passionate scientists I know.

My dissertation research would not have been possible without the assistance of numerous of undergraduate scientists. I am immensely grateful to Annika Greenleaf, Carolyn Burtt, Stuart Farnham, Madeline Bondy, and Maria Ostapovich, for their assistance and company in the field. I have never ceased to be impressed by the work ethic of each of these students, and I am so proud of the scientists that they have become.

A special thanks to the ecologists at The Trustees of Reservations for supporting me in my research. I would like to especially acknowledge Dr. Julie Richburg, who mentored me during my NSF internship in 2020, and Russell Hopping, who granted me permission to work at Appleton Farms in 2017, and helped me plan my most of my field work.

I would like to thank many of the friends I have made as a graduate student at Tufts (including Sarah Gilliand, Dr. Jenna Whalen, Dr. Brenna Gormally, Dr. Cassandra Donatelli, Dr. Natalie Kerr, Dr. Eric Scott, Nick Rosenburger, and Dr. Rachael Bonoan) for making my time a Tufts memorable. I am especially grateful to have met Gabriela Garcia, who has been a constant ally, and has grown to be one of my best friends. She is, without a doubt, one of the most amazing scientists I know.

Most of all, I would like to thank my family and closest friends for their unconditional love and support. Greg Mathieu and Altair Alonso, two of my closest friends, have supported me in innumerable ways. I am also incredibly grateful to my parents, Yolanda and Bruce Pugesek, for teaching me to love nature and encouraging me to pursue science. My dad, especially, has always been one of my biggest supporters. I am sure that if he were alive today, he would have been so excited to see me graduate. Lastly, to wonderful husband, Teagan Durtschi, I owe my most heartfelt thanks. He has been the best partner that anyone could ever hope to ask for, and, of course, the best field assistant.

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Chapter 1: Introduction

Genevieve Pugesek and Elizabeth E. Crone (2021) Introduction.

Insect populations have undergone dramatic declines over the last half-century (Sánchez-Bayo and Wyckhuys 2019, van Klink et al. 2020, Wagner et al. 2021). Bumble bees (*Bombus* spp.) are no exception to this general pattern and have, in fact, proven particularly sensitive to sudden declines (Bartomeus et al. 2013). Several once widespread species, including *B. affinis* and *B. terricola*, have all but disappeared from their historic habitat ranges (Colla & Packer 2008, Jacobson et al. 2018, Richardson et al. 2019, Graves et al. 2020). These widespread losses have been attributed to several factors which likely impact populations simultaneously. The most commonly cited drivers of declines include habitat loss, pesticide use, and climate change (Grixti et al. 2009, Williams et al. 2009, Goulson et al. 2008, Kerr et al. 2015, Soroye et al. 2020); however, exposure to pathogens and competition with invasive species have also been identified as threats (Szabo et al. 2012, Morales et al. 2013).

Regardless of the primary driver of bumble bee declines, managing and protecting existing habitat is essential for maintaining remaining populations. Retaining high-quality habitat, especially in areas where at-risk species are abundant, is critical to protecting source populations (Hodgson et al. 2011). Furthermore, maintaining habitat can benefit target populations by improving resilience to global stressors (Zaragoza-Trello et al. 2020); in the effort to mitigate species declines (especially in the face of global environment crises, like climate change), habitat management is one of the few tools available to conservation practitioners.

Maintaining habitat for bumble bees and other pollinators is also crucial to ecosystem health. Many species of crops and wildflowers are reliant on the pollination services provided by bees (Bauer 1983, Carreck and Williams 1998). The contribution of wild bees to crop pollination is valued at \$3,251$\cdot$ha^{-1}: although 80% of pollination services are provided by wild bees can be attributed to just 2% of species, many of these dominant crop pollinators belong to the genus

Bombus (Kleijn et al. 2015). In general, rates of visitation to flowering crops by wild bees are negatively correlated with distance to natural or semi-natural habitats (Ricketts et al. 2008). Habitat loss due to land-use change thus threatens the continued availability of pollination services.

Policies to enhance pollinator habitat often focus broadly on increasing the availability of floral resources, e.g., planting field margins (Wratten et al. 2012). There is some concern that these broad approaches could fail to adequately support rare or threatened species. Recently, there has been a push to implement more targeted habitat management strategies for threatened or endangered bumble bee species (e.g. U.S. Fish & Wildlife Service 2018). Although bumble bees, at least in contrast to other bee taxa, are relatively well-studied, there are many gaps in our understanding of particularly sensitive stages of the bumble bee life cycle, e.g., nesting and colony establishment, which prevent effective management action.

Most studies that characterize bumble bee habitat rely on collections of workers to quantify patterns of occupancy, abundance, or species richness (e.g. Purvis et al. 2020, Rosenberger and Conforti 2020). However, the resources and conditions necessary for bumble bee populations to persist can be difficult to infer from studies of worker abundance for two reasons. Firstly, bumble bees are social insects – workers live together in colonies, supporting one reproductive queen. Thus, it can be difficult to relate patterns of worker abundance to the mechanisms which drive them, because the colony, not the worker, is the unit of the population. Secondly, worker bumble bees spend most of their time outside the nest foraging. Studies of worker abundance are valuable in assessing foraging habitat (Walther-Hellwig and Frankl 2000), or, at broader spatial scales, monitoring range contractions and exploring landscape-level patterns (Colla and Packer 2008, Cameron et al. 2011). However, these studies do not identify all components of bumble bee habitat because bumble bees require sites to nest, mate, and overwinter, in addition to floral resources.

Although population ecology is largely unknown for many bee species, over the last two decades, researchers have made some strides in estimating demographic metrics for *Bombus* populations. For example, recent advances in the field of population genetics have greatly improved our ability to estimate *Bombus* nest densities in the field; by genotyping foraging workers and assigning workers to colonies, researchers may estimate the number of nests in a given area, as well as foraging ranges (Darvill et al. 2004, Knight et al. 2009, Goulson et al. 2010). By relating estimates of bumble bee nest densities to landscape configurations, molecular studies of bumble bees provided some of the first empirical evidence that *Bombus* populations are limited by floral resource availability (Knight et al. 2009). A few authors have even used molecular methods to estimate bumble bee vital rates, including the survival of family lineages from the summer worker to spring queen stages (Carvill et al. 2017). Studies of captive bumble bees, reared in the lab using wild-caught queens or purchased from suppliers, offer an additional opportunity to relate bumble bee vital rates to local floral resource availability or landscape configuration (Williams et al. 2012, Crone and Williams 2016, Malfi et al. 2019, Gervais et al. 2020). It is also fairly common to use captive bumble bee colonies to monitor the activity of workers (e.g., Osborne et al. 1999) or to assess the contents of pollen loads (Leonhardt and Blüthgen 2012) to infer foraging behavior. Thus, like studies which characterize patterns of worker abundance, the two methods discussed here have been primarily used to explore the impacts of landscape context and floral abundance.

Studies of Bombus nesting ecology

For many *Bombus* species, our knowledge of nesting habitat is derived entirely from incidental reports of nests, often describing the location of only a few nest sites (e.g., Ito et al. 1984, Gonzalez et al. 2004). From these anecdotal observations, bumble bee nesting habitat associations appear to be species-specific. Although bumble bee nests are frequently found beneath the ground in abandoned rodent burrows (Plath 1922), some species are mostly found nesting above the ground in tree cavities (Prŷs-Jones 2019) or on the ground surface under piles of vegetation (Hines et al. 2007). Though nest sites have been encountered in a variety of landscape contexts (Rau 1941, Sakagami and Katayama 1977, Hines et al. 2007), backyard encounters are a primary source of observations of nesting bumble bees (e.g., Fusell and Corbet 1992, Lye et al. 2011). As such, many reports of nesting bumble bees describe nests located in artificial structures, including birdhouses, compost piles, mounds of old clothing, and cavities in rock piles or stone walls (Putnam 1864, Donavan and Macfarlane 1971, Fussell and Corbet 1992, Prŷs-Jones 2014).

Studies of *Bombus* nesting have often relied on the use of man-made nesting structures to evaluate patterns of habitat selection. By placing empty nest boxes in different locations or positions in the landscape, researchers can explore the environmental factors that impact nest-site selection. The success of these studies in attracting nesting queens to nest boxes is highly variable; authors report rates of occupancy anywhere from 3% - 33% (Lye et al 2011, Richards 1978), with higher rates of occupancy for above-ground nest boxes than nest boxes in other positions (Johnson et al. 2019, Strange unpublished data, as cited by Herndon 2020). *Bombus* spp. that use artificial nest boxes are not representative of the *Bombus* communities as a whole (Johnson et al. 2019, Herndon 2020). For example, late-emerging species, like *B. perplexus* and *B. appositus*, seem to be particularly well-represented in studies which rely on the use of artificial nest boxes (Johnson et al. 2019, Herndon 2020). The most obvious limitation of this method, however, is the fact that placement of artificial nest sites may not reflect availability of nesting substrate on the landscape.

Researchers also have relied on several indirect methods to study *Bombus* nesting ecology. The abundance of nest-searching queens, for example, is a popular proxy for preferred habitat, as queen bumble bees are fairly conspicuous when engaged in nest searching behaviors (Svensson et al. 2000, Kells and Goulson 2003, Lye et al. 2009, O'Connor et al. 2017). A few researchers have leveraged molecular techniques to approximate colony locations using the centroid of sister worker bee locations (Redhead et al. 2015). Nonetheless, these approximations may be inaccurate because worker bumble bees may forage more than a kilometer from nest sites (Walther-Hellwig and Frankl 2000) and because many colonies may be represented only by a few individuals (Darvill et al. 2004).

Structured surveys of *Bombus* nests are an underused and underappreciated method of locating bumble bee nests, likely because many researchers consider locating *Bombus* nests directly to be prohibitively difficult (Liczner and Colla 2019). Only a handful of studies have leveraged these methods, primarily relying upon trained researchers or citizen scientists to search for nest sites in a predefined area (Osborne et al. 2008b, O'Connor et al. 2012, O'Conner et al. 2017). To circumvent some of the challenges in using visual surveys to located cryptic nest sites, researchers have also tried using thermal cameras or trained dogs to locate nests, but these methods are not generally any more successful than relying on volunteers (Waters et al. 2011, O'Connor et al. 2012, Roberts et al. 2020).

Regardless of methodology, there is considerable disparity in research addressing *Bombus* nesting across the northern hemisphere. The nesting ecologies of the United Kingdom's six most common bumble bee species are certainly the most-well studied, primarily due to the efforts by researchers in the U.K. and organizations like the Bumblebee Conservation Trust to engage citizen scientists in monitoring programs (e.g., Fusell and Corbet 1992, Osborne et al. 2008b, Lye et al. 2011). In the U.K., bumble bee nest densities tend to be high in gardens, hedgerows, and forest edges, though bumble bee nests are also found in grasslands and forests (Osborne et al. 2008b, O'Connor et al. 2017). It is unclear if the patterns observed in the United Kingdom can be extrapolated to other landscapes and other *Bombus* species, as bumble bee nesting habitat associations are species-specific and do not seem to be generalizable across subgenera (Lye et al. 2012, Liczner and Colla 2019). Further research is certainly necessary to evaluate the nesting ecology of bumble bee species native to regions outside of Europe.

Studies of Bombus overwintering ecology

Like our understanding of nesting habitat, our understanding of Bombus overwintering habitat is derived primarily from anecdotal observations (e.g., Sladen 1912, Plath 1927). Queen bumble bees are typically found overwintering underground in hibernaculum less than 8 cm below the ground surface, though reported depths range anywhere from 2-20 cm (Plath 1922, Alford 1969, Williams et al. 2019). Occasionally, overwintering queens have been found above the ground, e.g., in heaps of moss or other vegetation (Sladen 1912, Alford 1969). At broader spatial scales, overwintering queens of a few species, like *B. lapidarius*, have been associated with banks or slopes, whereas other species, like *B. vosnesenskii*, have been associated with areas near trees (Bols 1937, Williams et al. 2019). A few authors have noted, anecdotally, that queens seem to avoid overwintering in dense vegetation (though this observation may be more of an issue of visibility rather than preference). One *Bombus* species, *B. impatiens*, has been reported to overwinter colonially (Plath 1927, Townsend 1951, Szabo and Pengelly 1973). There is, however, no evidence to suggest that the tendency of bumble bees to overwinter colonially is common (Plath et al. 1927, Alford 1969).

Research Objectives

The two major goals of this dissertation are to 1) leverage the tools of population ecology to address knowledge gaps related to bumble bee nesting and overwintering ecology (Chapters 2, 3, and 5, as well as Chapter 6: Supplement) and 2) to explore the limitations of common methods of studying bumble bee habitat use and population health (Chapters 4 and 5).

In the second chapter of my dissertation, I explore species-specific patterns of nesting habitat use and quality for *Bombus* spp. native to the Northeastern United States by locating bumble bee nest sites directly. I conducted systematic, on-the-ground surveys of bumble bee nests to estimate nest densities in different land cover types. After finding nest sites, I monitored the reproductive output of colonies to relate to colony success to nest-site location. In addition to informing management guidelines for North American *Bombus*, this chapter addresses the general question of how habitat use and habitat quality align for bumble bees.

In the third chapter of my dissertation, I model the movement of queen bumble bees (*B. impatiens*) searching for nest sites to evaluate movement as an indirect metric of nesting habitat quality. I conducted on-the-ground surveys of flight paths, following queens searching for nests in hayfields, meadows, and forests. I related movement parameters calculated from flight paths to patterns to nesting-habitat associations observed in Chapter 2. Although researchers have relied on several indirect methods to study *Bombus* nesting (e.g., the abundance of nest-searching queens, see discussion above) as proxies for preferred habitat, to my knowledge, no study has leveraged the tools of movement ecology to infer nesting-habitat associations of bumble bee queens.

In the fourth chapter of my dissertation, I address some of the limitations of using commercial bumble bees as study subjects in studies of bumble bee foraging. Although commercially reared *Bombus*, *Apis*, and *Megachile* are considered model organisms in scientific research (Pitts-Singer and Cane 2011), the conditions experienced by commercial bumble bees in rearing facilities (and, later, in plastic nest boxes) are very different from those experienced by wild bumble bees. It is clear that commercialization has led to improved demographic performance of colonies reared in artificial conditions (Gosterit and Baskar 2016) – artificial rearing may have also led to behavioral changes in captive populations. In this chapter, I evaluated if wild workers behaved differently than workers from commercial colonies, at least, as they are typically used in research, an important first step in understanding whether studies of commercial bumble bees are representative of wild bumble bees.

In the fifth chapter of my dissertation, I explore whether lab-based estimates of bumble bee diapause survival are representative of mortality rates in natural populations. Diapause is an incredibly understudied stage of the bumble bee life cycle: only a handful of studies, almost all of which have been conducted in the lab, have estimated mortality rates for overwintering bumblebees. I conducted a field-based study to monitor the survival of diapausing queen bumble bees (*B. impatiens*). To compare my field-based estimates of overwinter survival to those produced by other studies, I conducted a meta-analysis of manuscripts that provide estimates of queen diapause survival. This study is the first of its kind, in that the queens monitored were overwintered in their natural hibernaculum. Thus, in addition to addressing the limitations of monitoring bumble bee vital rates in the lab, and outlining a novel method for monitoring bumblebee diapause survival, our study is the first to estimate overwintering mortality of natural bumble bee populations.

Chapter 2:
Contrasting effects of land cover on nesting habitat use and reproductive output for bumble bees.

Accepted to *Ecosphere*:

Genevieve Pugesek and Elizabeth E. Crone (2021) Contrasting effects of land cover on nesting habitat use and reproductive output for bumble bees.

Abstract

1. Understanding habitat quality is central to understanding the distributions of species on the landscape, as well as to conserving and restoring at-risk species. Although it is well-known that many species require different resources throughout their life cycles, pollinator conservation efforts focus almost exclusively on forage resources.
2. In this study, we evaluate nesting habitat for bumble bees by locating nests directly on the landscape. We compared colony density and colony reproductive output for *Bombus impatiens*, the common eastern bumble bee, across three different land cover types (hay fields, meadows, and forests). We also assessed nesting habitat associations for all *Bombus* nests located during surveys to tease apart species-specific patterns of habitat use.
3. We found that *B. impatiens* nested under the ground in two natural land cover types, forests and meadows, but found no *B. impatiens* nests in hay fields. Though *B. impatiens* nested at similar densities in both meadows and forests, colonies in forests had much higher reproductive output. In contrast, *B. griseocollis* tended to nest on the surface of the ground and was almost always found in meadows. *B. perplexis* was the only species to nest in all three habitat types, including hay fields. For some bumble bee species in this system, meadows, the habitat type with abundant forage resources, may be sufficient to maintain them throughout their life cycles. However, *B. impatiens* might benefit from heterogeneous landscapes with forests and meadows. Results for *B. impatiens* emphasize the longstanding notion that habitat use is not always positively correlated with habitat quality (as measured by reproductive output).

4. Our results also show that habitat selection by bumble bees at one spatial scale may be influenced by resources at other scales. Finally, we demonstrate the feasibility of direct nest searches for understanding bumble bee distribution and ecology.

Introduction

Ecologists often rely on habitat restoration and conversion projects to aid species recovery or enhance ecosystem services (Long 2009, Tonietto and Larkin 2018). However, implementing effective management strategies can be challenging without first identifying the resources and conditions necessary for target populations to persist (Thomas 1980). This general problem is illustrated by initiatives to address declines of pollinating insects. Most efforts to enhance pollinator habitat focus on protecting or enhancing foraging resources, i.e. floral abundance (Decourtye et al. 2010, Dicks et al. 2015). Although these efforts are laudable, increasing forage resources does not guarantee pollinator populations will be maintained. Insect populations may be limited by other factors, including the availably of host plants or nesting material (Potts et al. 2005, Flockhart et al. 2015). At the present time, our understanding of nesting ecology is insufficient to design effective management programs for many bee species. In thus study, we address this knowledge gap by identifying nesting habitat associations for bumble bees (*Bombus* spp.), an economically and ecologically important group of pollinators (Corbet et al. 1991, Carreck and Williams 1998).

Knowledge of nesting habitat requirements is a key limiting step in understanding the general ecology of *Bombus* and in conserving bumble bee populations. For example, the US Fish and Wildlife Service recently determined that it was “not prudent” to designate critical habitat for the endangered rusty-patched bumble bee (*B. affinis*), partially because the nesting and overwintering habitat requirements of this species are not well understood (US Federal Register 2020). Knowledge gaps like these exist, at least in part, because many researchers consider locating *Bombus* nests to be prohibitively difficult (Liczner and Colla 2019). Most studies describing nest locations are anecdotal, and describe the location of only a few nests (e.g., Gonzalez et al. 2004). A handful of researchers, mostly in the UK, have used systematic searches to estimate *Bombus* nest densities (e.g., Osborne et al. 2008b, O'Connor et al. 2017), but in these studies densities are confounded with search effort. Alternatively, researchers have used counts of nest-searching queens in different habitat types to study the spatial distribution of *Bombus* nests indirectly (Svensson et al. 2000, O'Connor et al. 2017). Thus far, both methods have shown little ability to discriminate use from detectability, or to separate nesting habitat use from quality (in terms of habitat-specific fitness, cf. Van Horne 1983). More detailed demographic data have been collected by researchers using molecular techniques to assign queens or foraging workers to colonies (Darvill et al. 2004, Knight et al. 2009, Carvell et al. 2017). Of these studies, a few have approximated nest locations from the locations of sibling worker bees (Lepais et al. 2010, Redhead et al. 2015, Carvell et al. 2017). However, though molecular studies of bumble bees are extremely valuable in exploring landscape-level patterns, they lack the precision to identify nest locations with the kind of resolution needed to provide meaningful guidance to improve nesting habitat. As such, conservation practitioners are left to operate under the assumption that bumble bees are generalists with no specific nesting habitat needs (cf. US Federal Register 2020).

In this study, we estimated key demographic variables for wild bumble bee populations in an effort to evaluate patterns of habitat use and habitat quality. Although *Bombus* nests are much harder to find than foraging worker bees, nest sites are not more difficult to find than nests of other taxa that have been widely studied using basic demographic tools, e.g., ground-nesting birds. At a set of study sites near Ipswich, Massachusetts, USA, we identified nesting habitat use by locating *Bombus* nest sites in three different land cover types. For the most commonly sighted species, *B. impatiens*, we estimated nest densities using mark-resight methods (Iles et al. 2019) to account for imperfect detection of nests and the possibility of habitat-specific differences in detection probability. We also monitored the reproductive output of *B. impatiens* nests we located in different land cover types by collecting gynes (female social insects with the potential to become queens) outside of nest entrances, as a way to assess habitat quality from a demographic perspective. In addition to demonstrating the feasibility of monitoring *Bombus* nests, our study is one of the few to systematically compare nesting habitat use for *Bombus* spp.

Materials and methods

Study sites

This research was conducted at three properties located just outside of Ipswich, Massachusetts, USA. Our fields sites were surrounded mainly by temperate deciduous forest and residential areas, although there is some agricultural activity in the area as well. Two of the properties, Appleton Farms (42°38'52.09"N, 70°51'1.01"W) and Appleton Grass Rides (42°38'33.26"N, 70°51'57.12"W), are mixed-use agricultural landscapes (Appendix 2.1). Appleton Farms is an active farm, whose dominant land cover types include hay fields and rangeland for cattle grazing, as well as more natural land cover types like wetlands, meadows, and forests. The adjacent site, Appleton Grass Rides, is dominated by forests, though a large meadow and several hay fields are maintained at the property. The third property, Greenwood Farms (42°41'35.77"N, 70°48'59.65"W), is a historic property located approximately 10 km from the other sites that is no longer used for agriculture (Appendix 2.1). This property is dominated by natural areas, including forests, marshes and grassy meadows. At the time of our study, all sites were owned and managed by The Trustees of Reservations, a non-profit land trust.

Nest density surveys

To estimate *Bombus* nest densities, we conducted systematic searches in hay fields, meadows, and forests. We limited our study to these areas, because prior to field work, we noted queen *Bombus* searching for nest sites in all three of these land cover types. The vegetation in hay fields, the most intensively managed land cover type, was dominated by a mixture of pasture grasses (e.g. *Phleum pratense, Dactylis glomerata*), legumes (*Medicago sativa*, *Trifolium* spp.), and weedy flowering plants (e.g., *Taraxacum* spp., *Daucus carota, Plantago* spp.). Most hay fields were harvested >2 times per year. Meadows and forests were managed less intensively, though meadows were mowed once annually to prevent succession. Primary vegetation in meadows included native grasses (e.g. *Schizachyrium scoparium*), herbaceous perennials (e.g. *Solidago* spp., *Rudbeckia* spp., *Asclepias syriaca*), and a handful of woody plant species (e.g. *Vaccinium angustifolium*, *Quercus* spp.). Invasive species (e.g. *Centaurea* spp.) were prevalent in a few areas at Greenwood Farms. Forest overstories were mostly hardwood or mixed hardwood-conifer, though there were a few stands of similarly aged pines (*Pinus* spp.) planted in rows at Appleton Farms and Appleton Grass Rides. Forests were typically open enough to walk through, though in some locations the forest understory could be dense. Forests at Appleton Farms and Greenwood Farms were noticeably fragmented compared to forests at Appleton Grass Rides (Appendix 2.1).

In 2018, we conducted nest density surveys at 30, 1500 m^2 plots. Each land cover type (hay field, meadow, forest) was represented by 10 plots, with 3-4 plots per land cover type located at each property. An additional three plots within hay fields were surveyed at Appleton Farms, as there were no hay fields at Greenwood Farms. To select plots, we first screened each property for potential survey areas by excluding areas whose primary vegetation consisted of invasive plants or that were marshy, as bumble bees will avoid nesting in poorly drained areas

(Lye et al. 2011). We selected plots within meadows vegetated primarily by grasses. We considered only forested areas adjacent to open areas (i.e., hay fields or meadows) when selecting forested plots, to better ensure bumble bees nesting in forests had similar access to floral resources to those nesting in open habitat. Many of the forested areas we surveyed shared an edge with surveyed meadows or hayfields. We avoided searching for nests in stands of planted pines, as these areas did not seem comparable to most forests within the study region. From these acceptable areas, we selected plots haphazardly, unless we had prior knowledge of *Bombus* nest locations, in which case plots were chosen randomly. In 2019, we selected 30 different plots in the same manner, for a total of 60 survey plots. We searched each plot for an hour-long period once a week, for a total of 4 sampling occasions per plot. We thus spent a total of 240 hours searching for nests during nest density surveys.

To locate nests, a single researcher (G. Pugesek) walked slowly through each plot, searching for worker traffic around nest entrances. Nests were located using both visual and auditory cues: by watching for queens or workers making a bee line toward the ground, or by listening for buzzing near the ground surface. Once a potential nest site was located, we confirmed the presence of a nest by waiting for at least four workers to exit or enter (Rao and Skyrm 2013). The first time a nest was located, the nest entrance was marked with an inconspicuous, numbered metal plant tag, and a single worker from each nest was collected to identify the colony to species (Iles et al. 2019). During subsequent searches, we recorded whether the nest was re-sighted to generate a capture history for each nest. Re-sighting a nest required the nest to be located using the same protocol, rather than by sighting the nest by memory or by locating an ID tag. Nest searches were carried out when *B. impatiens* colonies were large and when worker traffic at nest entrances was noticeable: from July 13th to August

15th in 2018 and July 12th to August 14th in 2019 (Iles et al. 2019). All surveys were conducted between 8:00 AM and 6:00 PM when the weather was clear.

Free searches for bumble bee nests

We supplemented our nest density surveys by conducting “free searches” for bumble bee nests, adapted from the methodology described by O’Connor et al. (2012). Our goal was to maximize the number of nests located, in order to improve our statistical power when making comparisons of habitat use across species. In contrast with nest density surveys, free searches for nests were haphazard, and were not implemented using mark-resight methods.

To conduct free searches, four different investigators walked haphazardly through natural areas at study sites, e.g., dry meadows, field margins, and forests, at their own pace, searching for bumble bee traffic around nest site entrances. After locating a potential nest site, the investigator confirmed the presence of a nest by waiting for the queen bumble bee to return with pollen baskets, or for at least two workers to either exit or enter the nest. The length of searches depended on weather conditions, but generally ranged from 2-3 hours. Searches were conducted at Appleton Farms and Grass Rides in 2018 and at all three properties in 2019. In 2018, we did not record the time spent searching each land cover type, though, in general, we spent more time searching for nests in open areas. In 2019, we spent approximately 150 hours searching for nests in meadows and 90 hours searching for nests in forests. Free searches for bumble bee nests took place prior to nest density surveys, between mid-April and mid-July, because the colonies of *B. griseocollis* and *B. perplexus* began to senesce prior to our last nest density surveys. Some of the same areas were searched during nest density surveys and free searches for nest sites; however, there was not much evidence that the probability we would locate nests during nest density surveys was influenced by prior knowledge of nest sites (see Appendix 2.2). All searches were conducted between 8:00 AM and 6:00 PM when the weather was clear.

Assessing nest site positions

The position of nest sites, e.g., whether the nest was located on the ground surface or below ground, was confirmed by visually inspecting nest site entrances, excavating nests, or by gently tapping the vegetation around nest sites (Kupchikova 1960). For most subterranean nests, the nest was clearly located underground, though removing some leaf material or vegetation was sometimes necessary to expose the entrance. For nests on the surface of the ground, in 2018, we conducted a similar visual inspection of the nest sites, i.e., we recorded whether worker bees seemed to be entering a tussock of grass or a rodent nest on the surface of the ground. In 2019, we excavated all surface nests after the colony had expired, or gently tapped the surface of the ground around the nest site with a stick, listening for buzzing workers (Kupchikova 1960).

Monitoring gyne output for each nest

We monitored *B. impatiens* nest entrances for activity of newly emerged gynes by watching nest entrances for 30 minutes, two times a week (see Appendix 2.3 for discussion of this protocol compared to others). Female workers were still prone to exit and enter the nest after colonies had switched into reproduction; thus, it was necessary to determine gynes via a visual assessment of size. Although bumble bee workers can be quite large, almost all workers were considerably smaller than queens. There were two occasions on which we were unable to easily assess whether a female was a small gyne or large worker – these individuals were released without being marked. We monitored colonies we had encountered during nest density surveys and free searches for nests, as described above, as well as three colonies encountered incidentally during field work to maximize our sample size. We ensured colonies had not expired prior to reproduction by monitoring each colony for activity (at least one worker either entered or exited the colony entrance during a one-hour period) prior to the surveys. Nest entrances were monitored from August 27th to October 23rd in 2018. No gynes were observed at field sites prior to this date. In 2019, we watched nest entrances from August 5th to October 30th, as we began observing gynes at nests in early August.

During each 30-minute survey period, gynes entering or exiting the nest were netted, cold-anesthetized, and marked with a unique number tag (Queen marking kit, BetterBee). At the end of each sampling period, captured gynes were released. Each colony was monitored until no workers or reproductives were encountered for four subsequent observation periods. Surveys took place between 9:30 AM and 5:00 PM; the time of day each nest was sampled was staggered. To quantify reproductive output for each nest, we tallied the total number of unique gynes encountered over the entire study period at each nest. We encountered no gynes at several nest sites; thus, using mark-recapture methods to estimate the total number of gynes produced by these colonies was not feasible. However, for colonies that produced gynes, we confirmed that the observed number of unique gynes encountered at each nest site was a strong positive predictor of the number of gynes produced by each colony as estimated using mark-recapture methods (R^2= 0.946, P < 0.001, see Appendix 2.4).

Statistical methods

B. impatiens nest densities were estimated using mark-resight methods. We used closed population models to estimate capture probabilities and nest densities from *B. impatiens* capture histories, because bumble bee nest site locations are fixed, and few colonies expired during our surveys. We implemented a Bayesian analysis using spatially stratified closed population models (Kéry and Royle 2015). Choosing a Bayesian analysis allowed us to estimate detection probability and account for variation in abundance between survey plots when estimating nest densities, a model structure that is not available in standard maximum likelihood mark-recapture software, e.g., program MARK (White and Burnham 1999). Models were run in JAGS using the R package jagsUI (Kellner 2019) for 300,000 interactions across three chains with a burn-in period of 10,000 iterations. We fit models including differences in detection among years and landcover type and performed model selection by estimating posterior model weights using indicator variables (Kuo and Mallick 1988, Kéry and Royle 2015). Estimates of posterior model weights are sensitive to model priors; thus, we set priors to posterior distributions calculated using the full model (Aitkin 1991, Kéry and Royle 2015). We verified models had converged by inspecting trace plots visually and confirming Gelman-Rubin convergence diagnostic values were less than 1.05 (Gelman and Rubin 1992, Kéry and Royle 2015). To determine if estimates of nest density differed across land cover types in each year of the study, we calculated a posterior distribution of the difference between means. If the 95% credible intervals of the difference overlapped with zero, we did not consider the means to differ.

As *B. impatiens* nests were not found in hay fields, we assumed the same detection probability for nests in hay fields and meadows when estimating nest densities. In mark-recapture models, the detection probability is the proportion of nests that are located in a single survey. In the absence of any nests in hay fields, it is impossible to statistically estimate this probability. However, it is reasonable to expect that our ability to detect nests would be similar between hay fields and meadows, because both land cover types are dominated by grasses and herbaceous plants. As we were able to locate other bumble bee species in hayfields, we believe it is reasonable to expect we would have been able to find *B. impatiens* nests as well, if they were present, at a similar detection rate to *B. impatiens* in meadows.

We assessed species-specific patterns of habitat use using multinomial logistic regression models, as there were more than two outcomes for the position of bumble bee nests (surface, tree stump, below ground), as well as general location (forest, hayfield, and meadow). Models were fit using the multinom function in the R package nnet (Venables and Ripley 2002; this and other R analyses were implemented in R version 3.6.1 [R Development Core Team 2019]). We pooled data from across years and study sites, and assessed nests located during free searches and nest density surveys, as well as nest sites found incidentally during other field work. Only nests that produced workers were included in this analysis, as we were unable to reliably identify queen bumble bees to species via observations in the field. We used separate models to compare nest position and surrounding land cover across bumble bee species. Species, our predictor variable, was categorized into three groups: *B. impatiens*, *B. griseocollis*, and other. *Bombus* species categorized as "other" (*B. perplexus*, *B. bimaculatus*, and *B. vagans*) were not encountered frequently enough to be assessed independently. Likelihood ratio tests, implemented using the command lrtest in package lmtest (Zeileis and Hothorn 2002), were used to compare univariate and null models. Post-hoc comparisons of estimated marginal means were made using the R package emmeans (Lenth 2020).

We analyzed differences in reproductive output, i.e., the total number of unique gynes encountered at each nest site, for *B. impatiens* using generalized linear models with negative binomial error distributions to account for overdispersion. Data were pooled across study sites due to low sample sizes. Models were fit using the R package MASS (Venables and Ripley

2002). Fixed effects included the surrounding land cover type (forest or meadow) and the year the colony was monitored (2018 or 2019); we also included an interaction between land cover and year. Candidate models included all possible combinations of variables and interactions (Table 2.1). Models were ranked and compared using Akaike's information criteria (AIC_C) corrected for small sample size using the AICc function in R.

Results

Nest densities

In two years, we located 29 *B. impatiens* nests during nest density surveys across all sites. *B. impatiens* nests were not found in hay fields, whereas in meadows and forests, we found 17 and 12 *B. impatiens* nests, respectively. We located bumble bee nests of other species as well (18 *B. griseocollis*, 1 *B. bimaculatus*, 1 *B. vagans*, and 2 *B. perplexus* nests), for a total of 51 nests (Appendix 2.5: Table S2.2).

We estimated capture probabilities for *B. impatiens* nests in meadows and forests, as no *B. impatiens* were found in hay fields. The model with the highest posterior model weight included land cover type as a covariate, M_c, with a posterior probability of 0.519. Searchers were less likely to detect nests found in forests (detection probability = 0.335) than in meadows (detection probability = 0.550). In contrast, the null model had a low posterior probability (0.174). There was not much evidence our ability to locate nests differed between survey years, as the models that included year, M_y, and both land cover type and year, M_{c+y}, as covariates also had low posterior probabilities (0.098 and 0.296, respectively). This covariate was thus excluded from further analyses.

In 2018 and 2019, estimated nest densities were consistently lowest in hay fields (Figure 2.1). In 2018, nest densities were lower in meadows than forests, with estimated nest densities of 4.28 nests · ha^{-1} in meadows and 7.58 nests · ha^{-1} in forests (a difference of -3.29 nests · ha^{-1}; 95% credible intervals [CRI]: -10.50 - 2.45). In 2019, we saw a larger difference in the opposite direction (+4.67 nests · ha^{-1}, 95% CRI: -0.61 - 10.23), with estimated nest densities of 7.41 nests · ha^{-1} in meadows and 2.74 nests · ha^{-1} in forests. Nest densities in meadows were lower in 2018 than 2019 (-3.13 nests · ha^{-1}, 95% CRI: -8.80 - 2.16), whereas nest densities in forests were higher in 2018 than 2019 (+4.84 nests · ha^{-1}, 95% CRI: -0.60 - 11.61).

Species-specific patterns of habitat use

A total of 123 bumble bee nests were located via a combination of free searches, nest density surveys, and incidental encounters (Appendix 2.5: Tables S2.2-S2.4). Most of the nests located were subterranean (61.9%) though many nests were also found on the surface of the ground, under tussocks of grass or within abandoned rodent nests (36.4%). Only two nests were found beneath decaying stumps (1.7% of nests found). Most nests sites were found in meadows (83.5%) as opposed to forests (14.8%) or hay fields (1.6%) (Figure 2.2), which was expected, given greater search effort in open natural areas during free searches.

Bombus species differed in terms of nest site position (χ^2= 85.69, df = 4, P < 0.001). Compared to *B. impatiens* and the other *Bombus* spp., a greater proportion (73.7%) of *B. griseocollis* nests were found on the surface of the ground. There was no significant difference in the proportion of nests found on the ground surface for *B. impatiens* (0.0%) compared to other *Bombus* spp. (7.0%; Appendix 2.6: Table S2.6). Similarly, there was no significant difference in the proportion of nests found of the below the ground for *B. impatiens* (98.0%) and other

Bombus spp. (84.6%; Appendix 2.6: Table S2.6), though a greater proportion of *B. impatiens* and the other *Bombus* spp. nested below ground compared to *B. griseocollis* (26.3%). The proportion of nests found under tree stumps did not differ significantly for *B. impatiens* (2.0%), *B. griseocollis* (0.0%), and other *Bombus* spp. (7.7%, Appendix 2.6: Table S2.6).

The use of different land cover types during nesting also differed across *Bombus* species (χ^2= 29.79, df = 4, P < 0.001). Relative to *B. impatiens*, for which 68.6% of nests were found in meadows and 31.4% of nests were found in forests, a greater proportion of *B. griseocollis* nests were found in meadows (98.3%) and a lesser proportion were found in forests (1.7%, Table Appendix 2.6: Table S2.7). No other comparisons were statistically significant (Appendix 2.6: Table 2.7).

Reproductive output

Over the course of two years, we monitored a total of 34 *B impatiens* colonies for gyne activity. In 2018, we monitored 8 *B. impatiens* colonies nesting in meadows and 9 *B. impatiens* colonies nesting in forests, and in 2019, we monitored 13 *B. impatiens* colonies nesting in meadows and 4 *B. impatiens* colonies nesting in forests. We collected and marked a total of 271 unique gynes.

Colony reproductive output was higher in forests than in meadows, and colonies reproduced more in 2019 than 2018 (Figure 2.3). The highest-ranking model included additive effects of both land cover and year (Table 2.1), though the model including only an effect of land cover (dAICc = 0.5) and the null model also ranked highly (dAICc = 1.0, for all other model comparisons, dAICc > 2). Colonies found in forests produced nearly three times as many gynes (14.0 gynes encountered · nest^{-1}) as colonies found in meadows (5.0 gynes encountered · nest^{-1}).

Colonies also produced about twice as many gynes in 2019 (10.9 gynes encountered · $nest^{-1}$) than in 2018 (5.5 gynes encountered · $nest^{-1}$).

Discussion

In the past, studies of *Bombus* nesting have been perceived as prohibitively labor-intensive, precluding effective habitat management for many species (Liczner and Colla 2019, US Federal Register 2020). In this study, we successfully located *Bombus* nests via on-the-ground surveys, and observed species-specific differences in nesting habitat use at two spatial scales, i.e., nest site position and surrounding land cover type. We also found that *Bombus* nest abundance was not always positively correlated with habitat quality as measured by reproduction. Together, these results emphasize the value and feasibility of studying nesting habitat in *Bombus* species.

To date, the consensus in the literature has been that bumble bees are nesting habitat generalists (Lye et al. 2012, Liczner and Colla 2019). This consensus exists in spite of past studies that have anecdotally reported differences in nest locations. For example, based on anecdotal reports, *Bombus* in tropical regions can be very specialized in their nest site selection (Gonzalez et al. 2004, Taylor and Cameron 2003). In a study of nesting habitat of *Bombus* communities the U.K., Lye et al. (2012) showed significant differences in the proportional use of different nest locations (subterranean, ground, above-ground) by different species. Although Lye et al. concluded that *Bombus* species were generalists because they all used more than one of these habitat types, differences in the frequency of use could reflect species-specific preferences. Past observations from North America have suggested that *B. impatiens* nests below ground (Plath 1922) and *B. griseocollis* nests on the ground surface (Harder 1986). In this study, we more rigorously document the differences in habitat use between these two taxa (see Figure 2.2 and Appendix 2.5). Together, these past reports and our current results indicate that it is premature to conclude that bumble bees are nesting habitat "generalists".

In addition, bumble bees may not be habitat generalists because habitat use is not equivalent to habitat quality (Van Horne 1983; Pulliam 1988). Our results highlight this well-known tenet of population ecology. Although we found a similar number of *B. impatiens* nests in forests (N = 12) and in meadows (N = 17) during nest density surveys, our results indicate that forests provide higher-quality nesting habitat, because *B. impatiens* colonies in forests produced more gynes than those in meadows (Figure 2.3). The importance of forests for *B. impatiens* is reinforced by Lanterman et al. (2019), who observed that queen *B. impatiens* tend to be more abundant in woodlands and woodland-field boundaries relative to other land cover types. The conclusion that forests are higher-quality habitat comes with the caveat that all of our study plots in forests were adjacent to areas where floral resources were abundant. Most colonies monitored were less than 10 meters from meadow edges, a short distance relative to worker bumble bee foraging ranges (Osborne et al. 2008a). Similarly, in other temperate regions, nesting bumble bees are more often associated with forest edges than interior forest habitat (Svensson et al. 2000, Osborne et al. 2008b). Understanding whether forests edges are higher quality habitat than forest interior could be a valuable area for future research. We also hypothesize that, although forests contribute to population viability of B. *impatiens* by providing nesting habitat, bumble bees need open habitat for foraging in order to persist at landscape scales.

Although we know that *B. impatiens* colonies nesting in forests produced more queens than those nesting in meadows, we do not know the mechanism for this difference. It seems unlikely that differences in reproductive output were driven by differences in the availability of floral resources, as the forested areas we surveyed were not adjacent to areas with unusually high floral abundance, and often shared an edge with surveyed meadows. We cannot exclude the possibility that detection probability is dependent on colony size. Because detection probability was higher in meadows, we may have been able to locate more small colonies in this land cover type. Bumble bee queen production increases with colony size (Crone and Williams 2016, Goulson et al. 2018b) and therefore a detection bias against small colonies in forests could lead to estimated lower average queen production in meadows. However, we believe it is unlikely that habitat-specific differences in reproductive output were driven by differences in detection probability, given that we were still able to locate small colonies in forests. In 2018, in particular, three of the four smallest colonies located during nest density surveys (i.e., colonies with the lowest worker activities around nest entrances when first encountered) were found in forests (Pugesek, personal observation). It may be that *B. impatiens* faced less competition for preferred nest sites in forests, as we encountered *B. griseocollis* more frequently in meadows (Figure 2.2, Appendix 2.5). If bumble bees are nest site limited (McFrederick and LeBuhn 2006, Inoue et al. 2008), competition may drive *B. impatiens* queens to accept lower quality nest sites to establish colonies at all. Perhaps the most likely explanation is simply that abiotic conditions in forests may have been more suitable for nesting *B. impatiens*. Tree cover can buffer air and soil temperatures and offer protection from the elements (Chen et al. 1995, Gaudio et al. 2017). Addressing these possibilities would be an interesting avenue for future research.

In addition to spatial variation, reproductive success of *B. impatiens* colonies also varied between years (Figure 2.3), a result that is consistent with research from other temperate regions. For example, a study monitoring the reproductive output of wild bumble bee colonies in the UK found that 71% of colonies produced gynes in 2010, whereas only 21.1% of colonies produced gynes in 2011 (Goulson et al. 2018b). Similarity, Richards (1978) found that 57% of *Bombus* colonies nesting in artificial domiciles produced queens and/or drones in 1970, whereas only 16% of colonies produced queens and/or drones the following year. Although the cause of this variation is not always clear (Goulson et al. 2018b), bumble bee population dynamics are likely impacted by factors such as precipitation and temperature (Goodwin 1995, Thomson 2016, Ogilvie et al. 2018).

Although we cannot explain many aspects of reproductive success, we can make some inferences about why *B. impatiens* nests were about equally abundant in meadows and forests, but *B. griseocollis* nests were found almost exclusively in meadows. In studies of vertebrate taxa, animal ecologists have often studied the relationship between habitat features and habitat selection at different spatial scales (e.g., Schaefer and Messier 1995, Stapp 1997). Johnson (1980) defines four orders of spatial habitat selection: the geographical range of a species (1st order selection), an individual's home range within that geographic range (2nd), the components of habitat within a home range (3rd), and the particular foraging items or nesting resources chosen (4th). In our study system, land cover type and nesting substrate correspond to resources at the 3rd and 4th orders of habitat selection, respectively (Johnson 1980). For bumble bees, the availability of nesting substrate (4th order habitat selection) seems to be an important driver of landcover type selection (3rd order habitat selection). For example, though subterranean nests were frequently encountered in both forests and meadows, surface nests were found almost exclusively in meadows, the only land cover type with suitable thatch for surface nest construction. Thus, for *B. griseocollis*, a surface-nesting species, distributions of nest sites seem to be driven by 4th order habitat selection. Lack of suitable nesting substrate could also explain why we found so few *Bombus* nests in hay fields relative to other land cover types during nest density surveys (Appendix 2.5): agricultural activities, like tilling or haymaking, are likely to destroy potential nest sites or drive away the animals whose burrows would be later used by nesting bumble bees. Understanding both 3rd and 4th order mechanisms of habitat selection is particularly relevant to translating landscape patterns to management action. For example, it might be easy to conclude that meadows and hayfields are similar habitat, but, at least from the perspective of ground-nesting species that build nests from thatch, this is not the case.

A final methodological lesson from our study is the importance of accounting for imperfect detection in estimating *Bombus* nest densities. Because detection probability was higher in meadows than forests in our study system, we might have underestimated the importance of forests for *B. impatiens* if we had searched study plots for nests only once. Other studies have compared nest densities between forests and grasslands without correcting for nest detection probabilities (Osborne et al. 2008b, O'Connor et al. 2017). However, even very small differences in detection probability across treatments or environmental gradients can lead to incorrect conclusions if unaccounted for (Archaux et al. 2012). The reality of imperfect detection also highlights the challenges, as well as the opportunities, of studying bumble colonies in the wild. We spent 160 hours searching 6 hectares of natural habitat for nests during nest density surveys, locating 29 *B. impatiens* nests. This effort may seem prohibitive to ecologists used to studying bumble bees by capturing foraging workers. However, we reiterate that this kind of intensity is similar to or less than the amount of labor required to locate nests of cavity- and ground-nesting birds, for which research on nesting ecology has had a long history of success (e.g., Saab et al. 2007). In studies of endangered or sensitive species, even greater search effort would be necessary to detect equal numbers of nests. However, in that case, direct surveys may still be worth the effort because they are non-invasive; in at least some cases, other methods of sampling workers to locate nest sites (e.g., tarsal clipping for genetic mark-recapture and radio-tagging) have negative effects on individual bees (e.g., Hagen et al. 2011, Mola et al. 2021).

For many species of bumble bees, especially those that are rare or threatened, habitat requirements during crucial life history events like nesting are poorly understood. Though the results presented here primarily represent common bumble bee species, our findings inform future efforts to monitor bumble bee populations by showcasing feasible strategies for collecting demographic data. Even for these common species, our research emphasizes that patterns of bumble bee nesting habitat use are species specific: for example, *B. griseocollis* would be more likely than *B. impatiens* to persist in landscapes that contained only meadows, but, because it is surface nesting, would be more susceptible to disturbances like haying or fire. Applying these methods to other landscapes, perhaps especially in regions where at-risk species are still locally common, could be a valuable next step in bumble bee conservation.

Acknowledgements

Funding for this research was provided by the Tufts University College of Arts and Sciences and the National Science Foundation (award DEB 1354022 to E. E. Crone). G. Pugesek led field work, and both G. Pugesek and E. E. Crone conceived ideas and designed methodology, conducted data analyses, and contributed to writing this manuscript. We would like to acknowledge Maria Ostapovich, Madeline Bondy, and Stuart Farnham for their assistance in the field. We also thank James Michielini for his assistance converting raw data into capture histories for gynes and completing the analyses described in Appendix 2.4, and Dr. Andy Royle for his assistance implementing spatially stratified closed population models. We thank The Trustees and Russell Hopping for their support. The authors declare no conflict of interest.

Tables

Table 2.1. AICc statistics for competing generalized linear models (family = negative binomial) used to estimate reproductive output of *B. impatiens* colonies.

Model abbr.	Effects	df	AICc	Model weight
M_{c+y}	*Cover + Year*	4	199.6	0.332
M_c	*Cover*	3	200.0	0.264
M_0	*Null*	2	200.6	0.203
M_y	*Year*	3	201.7	0.116
M_{c*y}	*Cover*Year*	5	202.3	0.085

Figures

Figure 2.1. Nest densities and associated 95% Bayesian credible intervals for *B. impatiens* across three different land cover types estimated using spatially explicit closed capture models.

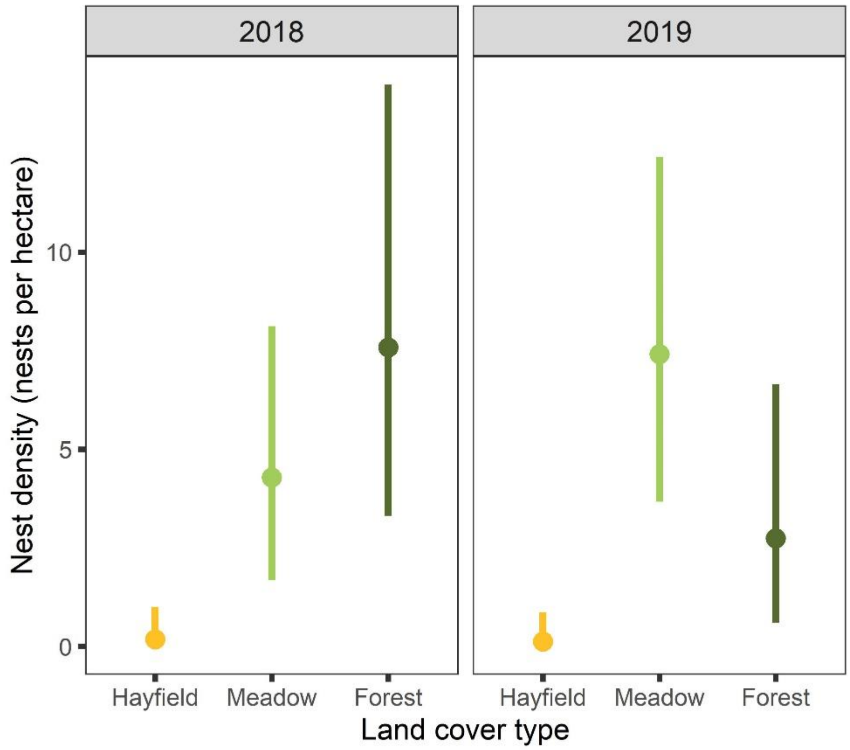


Figure 2.2. a) The position of nest sites and b) the land cover type where nests were found for *B. griseocollis* (B. grise.), *B. impatiens* (B. impat.), *B. bimaculatus* (B. bimac.), *B. perplexus* (B. perpl.), and *B. vagans* (B. vagan.). Letters (c and d) indicate statistical significance groups within each subpanel.

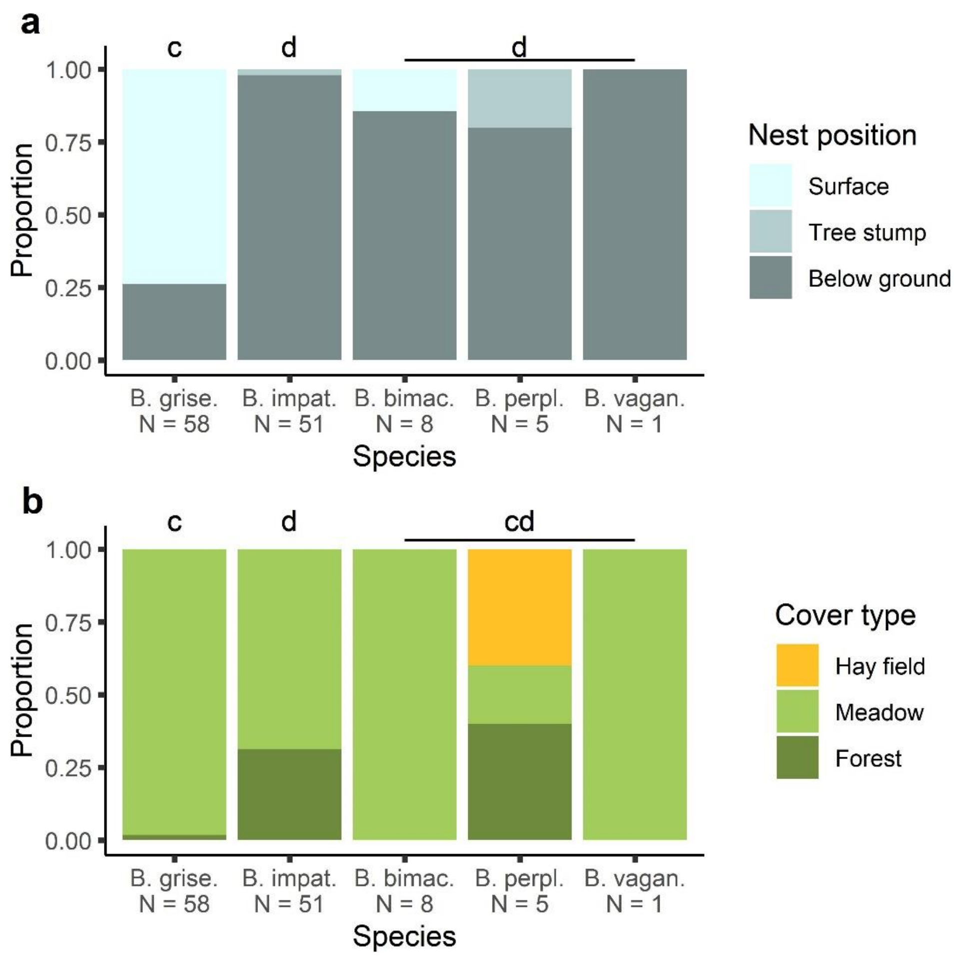
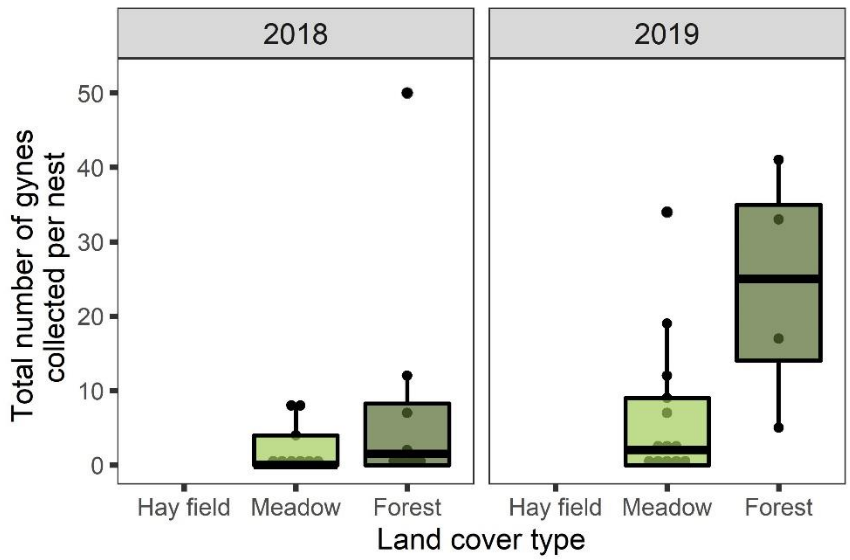


Figure 2.3. The total number of unique gynes encountered at *B. impatiens* colonies located in hayfields, meadows, and forests. As no *B. impatiens* colonies were found in hayfields, this landcover type was excluded from statistical comparisons.



Appendix 2.1

Figure S2.1. An aerial view of our study sites: (A) Appleton Farms (42°38'52.09"N, 70°51'1.01"W), (B) Appleton Grass Rides (42°38'33.26"N, 70°51'57.12"W), and (C) Greenwood Farms (42°41'35.77"N, 70°48'59.65"W). Images were created in ArcMap. Data sources include Esri, DigitalGlobe, GeoEye, i-cubed, USDA FSA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community.

Appendix 2.2

Behavioral effects on capture probabilities

Capture probabilities may differ between the first time an animal is located and subsequent captures, a phenomenon known as a behavioral effect. These effects commonly arise due to trap avoidance or trap happiness, when animals learn to avoid traps or learn to visit traps to consume baits (Wegge et al. 2004, Wilson et al. 2011). However, in our study system, behavioral effects may arise because surveyors remember the location of a nest after finding it for the first time or because trampling the vegetation around nest sites may alter the visibility of nest entrances, leading to higher capture probabilities during subsequent searches (Iles et al. 2019). Though searches of plots were conducted systematically, and surveyors located nest sites based on sightings of workers outside of nest entrances rather than on prior knowledge of nests, it is possible prior knowledge of nest sites may have led to unconscious bias during searches.

We explored whether behavioral effects impacted capture probabilities of nests using two different methods. We first fit Huggins closed capture models (Huggins 1989) to capture histories for *B. impatiens*. Models were run in program MARK using the RMark library (Laake 2013) in R version 3.6.1 (R Core Team 2018). Two separate models were tested: a null model with a constant value for capture probability across all nests (M_0), and a model for which behavioral effects on capture probability are accounted for (M_b) (see Table 2.1). Models were ranked and compared using Akaike's information criteria corrected for small sample size (AIC_C).

These capture-recapture analyses are unable to account for the fact that, of the 51 *Bombus* nests encountered during mark-resight surveys, 25 nests had been located at least once prior to the start of surveys. (During mark-resight surveys, we also failed to encounter 4 nest sites with known locations.) Thus, we conducted a second, *ad hoc* analysis to account for all prior knowledge of nest sites. We used a generalized linear model (binomial family, logit link) to test whether the probability of finding nest sites during mark-resight surveys differed between nests that had previously been located during field work (e.g., during free searches or previous mark-resight surveys), and those for which we had no prior knowledge. All *Bombus* nests located during nest density surveys were included in this analysis; thus, we also tested if the probability of finding a nest site was affected by the species of bumble bee using the nest site (categorized as *B. impatiens* vs. other *Bombus spp*.). Prior to analyzing data, we removed the last two sampling occasions from our data set for *Bombus* species with shorter colony lifespans (e.g., *B. griseocollis*, *B. perplexus*), because many colonies had senesced by that point. Otherwise, data were pooled across sampling occasions. Type II marginal hypothesis tests implemented using the Anova function in package car were used to determine the significance of predictor variables (Fox and Weisberg 2019).

Results

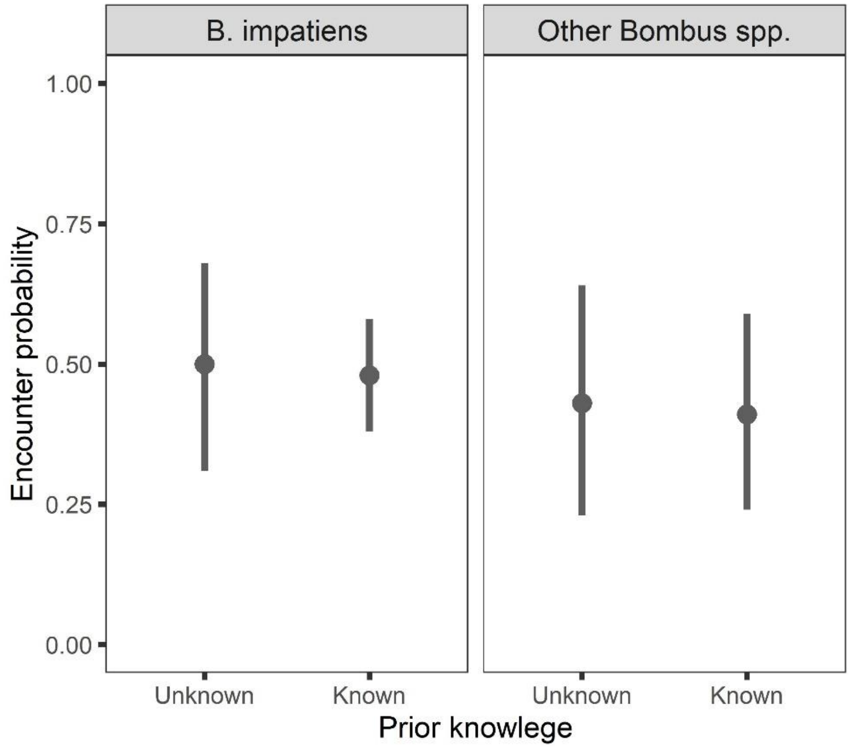
There was not much evidence our ability to locate nests was affected by prior knowledge of nest sites. Of the two closed population models tested, the null model ranked the highest according to Akaike's information criterion (AIC*c*), followed by the model which included a behavioral effect (see Table S2.1).

Nests encountered during previous field work (free searches, nest density surveys, etc.) were as likely to be sighted during nest density surveys as unknown nests (χ^2 = 0.020, df = 1, *P* = 0.889, Figure S2.2). The probability of detecting a nest did not differ statistically between *B. impatiens* and other *Bombus* species (χ^2 = 0.748, df = 1, *P* = 0.387, Figure S2.2): the detection probability of an individual *B. impatiens* nest was 49-50% on any given search, whereas the detection probability for an individual nest of another *Bombus* species was 41-43% on any given search. Our findings are similar to those of Iles et al. (2019), who used similar methods to locate bumble bee nests and found no evidence of behavioral effects while searching for nest sites.

Table S2.1. Model selection results for competing Huggins closed capture models used to estimate *B. impatiens* nest densties and detection probabilites.

Model Abbr.	Effects	No. Parameters	AIC_c	ΔAIC_c	Model Weight
M_0	*Null*	1	158.74	0	0.715
M_b	*Behavior*	2	160.58	1.84	0.285

Figure S2.2. The impact of prior knowledge on the probability of encountering *B. impatiens* nests and the nests of other *Bombus* spp.



Appendix 2.3

Methods for monitoring reproductive output of bumble bee colonies

In 2018, we explored several approaches for monitoring the number of new gynes produced by each colony. We first staked a headless soil emergence trap over the entrance of each nest (BugDorm), leaving a small opening fit with a gyne excluder at the top and bottom of the tent. The entrances of the excluders were too small for gynes to pass through but allowed for the passage of most worker bumble bees (6.3 mm in height). Unfortunately, workers seemed unable to find their way out of the trap. We then minimized the size of the emergence trap, instead covering each colony entrance with a 175 mL plastic container, painted white, with an opening fit with a gyne excluder (Figure S2.3). Workers were able to pass through this entrance with no issues. However, this trap could only cover a small area surrounding a nest entrance. Because many colonies have multiple nest entrances, we were concerned that gynes would emerge from a different exit of the colony and would bias our results.

Given our concerns with emergence-trap methods, we monitored gyne activity at nests by watching nest entrances for 30 minutes, 2 times a week (as described in the main text of this paper). We used raw counts of gynes at nest entrances to estimate reproductive output of colonies (see main manuscript and Appendix 2.2). Similar methods have been employed by Goulson et al. (2018a) and Goulson et al. (2018b), who monitored wild bumble bee nest entrances for gyne activity using cameras (Goulson et al. 2018b) or by relying on observations made by citizen scientists (Goulson et al. 2018a).

Figure S2.3. a) The side view and b) front view of small emergence traps. Traps were held to the ground using a brick.

Appendix 2.4

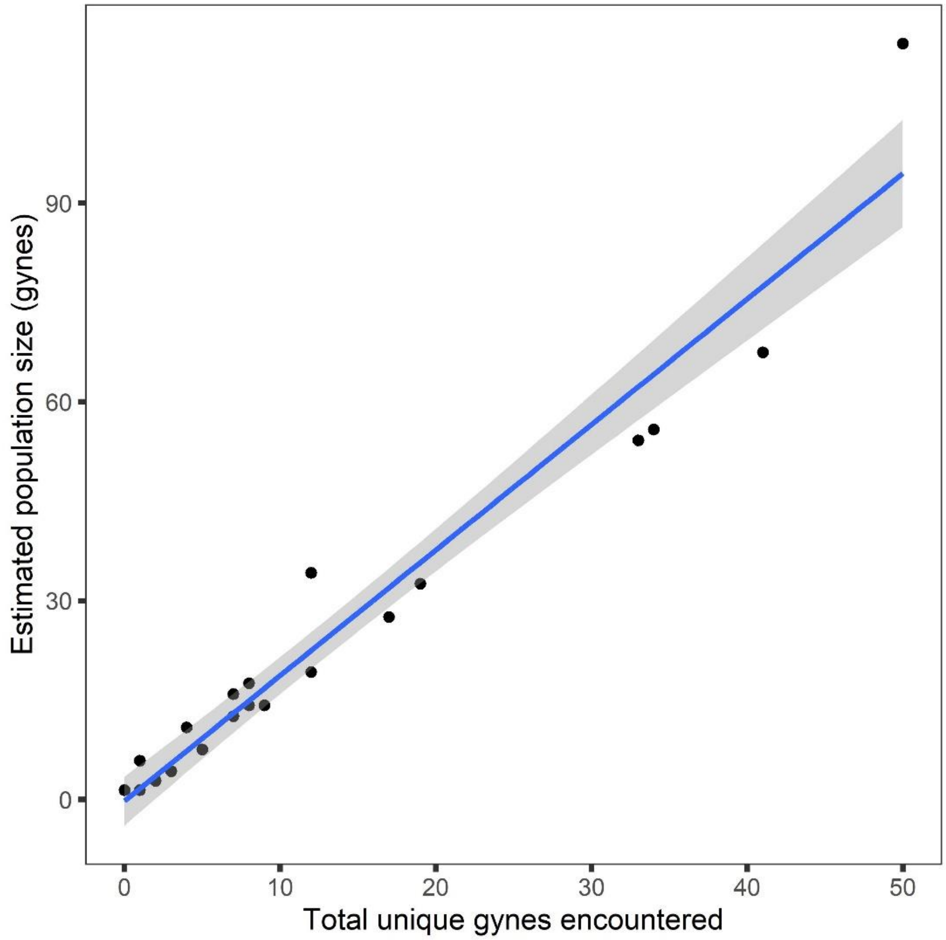
Mark-recapture analysis of gynes

Using mark-recapture methods, we tested whether counts of unique gynes encountered were representative of total number of gynes produced by each colony. We first estimated detection probability and apparent survival of gynes using Cormack-Jolly-Seber models. Models were run in program MARK (White and Burnham 1999) using the R package RMark (Laake 2013). Apparent survival does not differentiate mortality and emigration; however, given the natural history of bumble bees, most gynes are likely dispersing from natal nest sites to either mate or overwinter. A capture history for each marked gyne was generated using surveys of gynes exiting and entering nests. In 2018, we completed additional surveys of nest sites to increase the likelihood we would recapture individuals: each nest site was surveyed an additional 1-2 times per week in 2018. We fit mark-recapture models to this data set assuming constant detection probability (p) and apparent survival (ϕ) of gynes across all nest sites. We used the transformation $-1/\ln(\phi)$ to estimate residence time of gynes in their natal colonies.

For colonies at which gynes were encountered (23 of 34 colonies monitored), we used a second analysis to estimate total population size using Cormac-Jolly-Seber models. These models used fixed values of p and ϕ from the step-1 analysis of all colonies combined and were then used to estimate the total number of gynes produced by each colony (there were typically too few recaptures to estimate all parameters separately for each nest). We then compared mark-recapture estimates to the number of individual gynes observed at each nest using a linear model. We used type II marginal hypothesis tests implemented using the Anova function in R package car to determine the significance of predictor variables (Fox and Weisberg 2019).

Gynes remained at nests sites for 1.26 (95% CI: 1.19 - 1.53) days after emergence from nest sites, transformed from a daily apparent survival rate of 0.45 (95% CI: 0.41 - 0.49). When gynes were available for capture, probability of detection was 0.76 (95% CI: 0.69 - 0.82). Pooled together, colonies were estimated to produce 546 gynes (95% CI: 504 - 591). The number of unique gynes encountered at each nest site was predictive of mark-recapture estimates of the number of gynes produced by each colony ($F_{1,21}$= 384.95, R^2= 0.946, P < 0.001, Figure S2.4). The slope of the regression line was 1.894, indicating colonies produce nearly twice as many gynes as those encountered at nest sites. Colonies where no gynes were encountered likely produced few gynes, if any, as the intercept of the regression line did not differ significantly from zero (t = -0.144, P = 0.887).

Figure S2.4. The relationship between the number of unique gynes we encountered at each nest site and mark-recapture estimates of the number of gynes produced by each colony.



Appendix 2.5

Table S2.2. The number of bumble bee nests found during systematic searches of hay fields, meadows, and forests at three properties (Appleton Farms [AF], Appleton Grassrides [AG], and Greenwood Farms [GF]) located just outside of Ipwich, MA during the summer of 2018 and 2019.

Land cover	*Bombus* species	Number of nests encountered (AF)		Number of nests encountered (AG)		Number of nests encountered (GF)	
		2018	2019	2018	2019	2018	2019
Hay fields	*B. impatiens*	0	0	0	0	-	-
	B. bimaculatus	0	0	0	0	-	-
	B. griseocollis	0	0	0	0	-	-
	B. vagans	0	0	0	0	-	-
	B. perplexus	0	1	0	1	-	-
Meadows	*B. impatiens*	4	5	2	4	0	2
	B. bimaculatus	1	0	0	0	0	0
	B. griseocollis	3	2	3	3	0	6
	B. vagans	0	0	0	0	1	0
	B. perplexus	0	0	0	0	0	0
Forests	*B. impatiens*	1	1	7	1	1	1
	B. bimaculatus	0	0	0	0	0	0
	B. griseocollis	0	1	0	0	0	0
	B. vagans	0	0	0	0	0	0
	B. perplexus	0	0	0	0	0	0

Table S2.3. The number of bumble bee nests found during free searches of different land cover types at three properties (Appleton Farms [AF], Appleton Grassrides [AG], and Greenwood Farms [GF]) located just outside of Ipwich, MA, in 2018 and 2019.

Land cover	*Bombus* species	Number of nests encountered (AF)		Number of nests encountered (AG)		Number of nests encountered (GF)	
		2018	2019	2018	2019	2018	2019
Meadows	*B. impatiens*	5	5	3	5	-	5
	B. bimaculatus	1	0	3	3	-	1
	B. griseocollis	3	1	8	25	-	6
	B. vagans	0	0	0	0	-	0
	B. perplexus	0	0	0	2	-	0
Forests	*B. impatiens*	0	1	1	2	-	1
	B. bimaculatus	0	0	0	0	-	0
	B. griseocollis	0	0	0	0	-	0
	B. vagans	0	0	0	0	-	0
	B. perplexus	0	0	0	1	-	1

Table S2.4. The number of bumble bee nests found incidentally in different land cover types at three properties (Appleton Farms [AF], Appleton Grassrides [AG], and Greenwood Farms [GF]) located just outside of Ipwich, MA, in 2018 and 2019.

Land cover	*Bombus species*	Number of nests encountered (AF)		Number of nests encountered (AG)		Number of nests encountered (GF)	
		2018	2019	2018	2019	2018	2019
Meadows	*B. impatiens*	2	3	0	3	0	0
	B. bimaculatus	0	0	0	0	0	0
	B. griseocollis	0	1	1	2	0	2
	B. vagans	0	0	0	0	0	0
	B. perplexus	0	0	0	0	0	0
Forests	*B. impatiens*	0	0	2	0	0	0
	B. bimaculatus	0	0	0	0	0	0
	B. griseocollis	0	0	0	0	0	0
	B. vagans	0	0	0	0	0	0
	B. perplexus	0	0	0	0	0	0

Appendix 2.6

Table S2.6. Post-hoc comparisions between *Bombus* species (*B. griseocollis* [B. gris.], *B. impatiens* [B. imp], and other *Bombus* spp. [B. spp]) by nest position.

Nest position	Contrasts	Estimate ± SE	t-ratio	*P*
Below ground	*B. gris. vs. B. imp*	-0.72 ±0.06	-11.638	<0.001
	B. gris vs. B. spp	-0.58 ± 0.12	-5.033	0.006
	B. imp vs. B. spp	0.13 ± 0.10	1.302	0.440
Surface	*B. gris. vs. B. imp*	0.74 ± 0.06	12.633	<0.001
	B. gris vs. B. spp	0.66 ± 0.09	7.009	0.001
	B. imp vs. B. spp	-0.08 ± 0.07	-1.041	0.581
Tree stump	*B. gris. vs. B. imp*	-0.02 ± 0.02	-1.010	0.598
	B. gris vs. B. spp	-0.08 ± 0.07	-1.041	0.581
	B. imp vs. B. spp	-0.06 ± 0.08	-0.744	0.748

Table S2.7. Post-hoc comparisions between *Bombus* species (*B. griseocollis* [B. gris.], *B. impatiens* [B. imp], and other *Bombus* spp. [B. spp]) by surrounding land cover type.

Land cover	Contrasts	Estimate ± SE	t-ratio	*P*
Meadow	*B. gris. vs. B. imp*	0.30 ± 0.07	4.413	0.011
	B. gris vs. B. spp	0.27 ± 0.12	2.200	0.149
	B. imp vs. B. spp	-0.03 ± 0.14	-0.204	0.977
Forest	*B. gris. vs. B. imp*	-0.30 ± 0.07	-4.221	0.011
	B. gris vs. B. spp	-0.13 ± 0.08	-1.433	0.435
	B. imp vs. B. spp	0.17 ± 0.11	1.556	0.355
Hay Field	*B. gris. vs. B. imp*	<0.001 ± <0.001	0.343	1.000
	B. gris vs. B. spp	-0.14 ± 0.09	-1.154	0.344
	B. imp vs. B. spp	-0.14 ± 0.09	-1.154	0.344

Chapter 3:
Movement of bumble bee queens as an indirect metric of nesting habitat quality.

In preparation:

Genevieve Pugesek and Elizabeth E. Crone (*In prep*) Movement of bumble bee queens as an indirect metric of nesting habitat quality.

Abstract

1. Movement of foraging animals has been commonly used as an indirect index of resource availability or habitat quality, as animals are predicted to move in a way that maximizes the time spent in areas where resources are abundant. Here, we model the movement of nest-searching bumble bee queens (*B. impatiens*) to explore movement as an indirect metric of habitat quality.
2. At a farm in eastern Massachusetts, we recorded the flight paths of nest-searching queen bumble bees in three land cover types (hayfields, meadows, and forests). These landcover types represent both habitat and non-habitat for nesting bees, as previous research at this field site shows *B. impatiens* does not nest in hayfields but nests in both forests and meadows at relatively high densities.
3. We found that queen *B. impatiens* searching in meadows and forests exhibited behaviors indicative of area-restricted search: in forests and meadows, nest-searching queen bumble bees had smaller step lengths than in hay fields. Assuming bee movement during nest searching can be modeled as a correlated random walk, we calculated a diffusion coefficient of 0.366 $m^2 \cdot s^{-1}$ for queens searching for nest in hay fields, and diffusion coefficients of 0.119 $m^2 \cdot s^{-1}$ and 0.224 $m^2 \cdot s^{-1}$ for bees in searching for nests in meadows and forests, respectively.
4. Our research demonstrates a novel application of on-the-ground surveys of movement to assess nesting habitat for bumble bees, in addition to showcasing movement of nest-searching queen bumble bees as potential indicator of future bumble bee nest site locations.

Introduction

Ecological theory predicts that in heterogenous environments, animals will move in a way that maximizes the amount of time spent in areas where resources are abundant (Turchin 1998). This behavior, also known as area-restricted search, is characterized by larger turning angles and shorter move lengths (Figure 3.1). Studies of movement behavior using remote tracking methods have proven particularly valuable in identifying patterns of habitat use for marine vertebrates (Suryan *et al.*, 2006; Freitas *et al.*, 2008). In addition, as area-restricted search is thought to be induced by prey capture (Kareiva & Odell, 1987), animal movement has also been used to evaluate resource abundance indirectly (Chimienti *et al.*, 2017). Although area-restricted search is primarily regarded as a foraging strategy, models of animal movement have also been used to evaluate behavioral responses of Lepidoptera (butterflies and moths) to non-foraging resources (e.g., adults responding to larval host plant availability; Schultz et al. 2012a, Brown et al. 2017, Crone et al. 2019, Evans et al. 2020). In this study, we leverage similar methods to quantify the nest-searching strategy of bumble bee queens (*Bombus impatiens*), as an indirect metric of nesting habitat quality.

For bumble bees, identifying indirect metrics of nesting habitat quality is critical, because we understand so little about nesting habitat requirements. Many bumble bee species have undergone local extinctions, with 23 species listed as vulnerable, threatened, or endangered by International Union for Conservation of Nature (IUCN). Conservation guidelines for endangered bumble bees, e.g. *B. affinis*, emphasize the need to manage or maintain nesting habitat (U.S. Fish & Wildlife Service, 2018). However, for most bumble bee species, our understanding of nesting habitat is limited or informed primarily by anecdotal observations of nests (Plath, 1922; Gonzalez *et al.*, 2004). Although a handful of researchers have conducted formal surveys of bumble bee nests (O'Connor et al. 2017, Iles et al. 2019), most of these studies can be incredibly time intensive. In recent years, researchers have explored several alternative techniques for locating bumble bee nests, including the use of trained detection dogs and thermal cameras to locate nest sites (Waters et al. 2011, O'Connor et al. 2012, Roberts et al. 2020), with only limited success.

As an alternative to locating nest sites directly, past studies have used the abundance of nest-searching queen bumble bees as an indirect metric of nesting habitat preference (Svensson et al. 2000, Lye et al. 2009, O'Connor et al. 2017, Lanterman et al. 2019). Nest-searching queens are more conspicuous than bumble bee nests, and move in a highly stereotyped, zig-zag flight pattern that makes them easy to discriminate from foraging queens (Svenssen et al. 2000). Abundances of nest-searching queen are positively, albeit weakly, correlated with nest densities (O'Connor et al. 2017). There is some concern, however, that queen abundance may be an unreliable indicator of habitat quality if the presence of nest-searching queens is more indicative of an inability to locate a suitable nest site than a preference (O'Connor et al. 2017). Furthermore, it is possible high prevalence of parasites could also lead to inflated counts of nest-searching queens. For example, infection by the nematode *Sphaerularia bombi* can prolong nest-searching behavior of queens (Lundberg & Svensson, 1975); by the end of spring, this nematode can be quite prevalent (Macfarlane & Griffin, 1990).

Monitoring the movement of nest-searching queens offers a potential alternative to locating nests directly or estimating nest-searching queen abundance. For other taxa, immigration rates estimated from local movement are subject to less temporally variability than estimates of abundance, suggesting indicators of habitat quality based on movement are more reliable (Bélanger & Rodríguez, 2002). Furthermore, movement behavior may be a better indicator of habitat quality than abundance in areas adjacent to high-quality habitat, where animals may be abundant due to spill-over, or in situations where habitat selection is impacted by competition for resources. As an added benefit, movement data can be generated from observations of relatively few individuals (Lode 2000, Fauchald and Tveraa 2003), which is ideal in landscapes where target species are rare.

In this study, we tracked the movement of nest-searching bumble bee queens at a single site (Appleton Farms, Ipswich, Massachusetts, USA), and calculate diffusion coefficients as an integrative measure of nest searching strategy. As the nesting ecology of *B. impatiens* is relatively well understood (Iles et al. 2019, Lanterman et al. 2019), ours is an excellent study system for confirming the validity of this method. At this study site, *B. impatiens* are known to nest in meadows and forests at this study site but tend not to nest in hayfields (Iles *et al*., 2019; Pugesek and Crone, *in press*). Furthermore, although *B. impatiens* nests at comparable densities in both of these land cover types, colonies found in forests have higher reproductive output than colonies found in meadows, suggesting forests provide higher-quality nesting habitat (Pugesek and Crone, *in press*). As such, we expected that nest searching queens would move more slowly within habitat (meadows and forests) than in non-habitat (hay fields). We also expected that queens would move more slowly in forests (the higher-quality habitat) than in meadows.

Materials and Methods

Flight paths

In the spring of 2018 (between April 26th and May 16th), we mapped flight paths of queen bumble bees by following focal individuals on foot from a distance (>1m) and dropping a numbered flag at locations where the queens inspected an area on the ground as a potential nest site. Nest-searching queens fly slowly, and thus it was possible conduct on-the-ground surveys of movement (Cresswell 1995; Schultz 1998). As we followed queens, we noted the time the queen began each inspection, as well as the time at which the queen stopped her inspection. On a few occasions, the bee remained at a potential nest site for over a 5-minute period, after which the flight path was ended. The locations of flags were recorded via GPS (Trimble Geo7x), with two subsequent locations corresponding to a single movement step (see Figure 3.2 for example flight paths). Assays of the same individual across different land cover types were assessed as separate flight paths (with one flight path representing movements within only one land cover type), and all flight paths shorter than two steps were excluded from further analyses.

To avoid repeated sampling of the same individual, after each flight path was collected, we attempted to capture and mark the focal individual a colored paint marker (Sharpie paint, oil-based marker). Capturing individuals after the flight path was completed was not always possible: we only captured and marked 19 of 95 individuals followed. However, we are not concerned repeated sampling of unmarked individuals occurred, given marked individuals were rarely resighted (a marked individual was encountered only once). In other study systems, recapture rates of tagged bumble bee queens tend to be very low, with resightings of only 16% of tagged queens (Stenström and Bergman 1998).

Statistical Analyses

To quantify bumble bee movement, we conducted two sets of analyses using flight path data. First, we estimated parameters to calculate the diffusion coefficient, D, for each queen using the correlated random walk approach (i.e., average step length, squared step length, step time, and cosine of the turning angle, see Turchin, 1998; Brown & Crone, 2016). We use diffusion as an integrative metric of the rate at which animals move through habitat while searching for nests, even though nest-searching bumble bees do not "diffuse" at longer time scales. For bumble bees, bouts of nest-searching are often punctuated by longer-distance flights; previous research has indicated these insects can fly large distances in a single stretch: spring queens have been observed to "mass migrate" across the large bodies of water (Fijen 2020), and have been sighted over 50 km away from shore (Mikkola 1984). These behavioral modes are not captured by our data. Our estimates of diffusion coefficients thus represent the rate at which areas were explored during nest searching, rather the rate at which spring queens diffuse through the landscape.

Movement parameters for each bee were estimated using linear mixed effects models with a random effect of focal bee ID, implemented using the lme4 package in R (Bates *et al.* 2014). We did not transform the data prior to fitting models, because, according to Jensen's inequality, transformation will alter coefficient values. After we obtained an estimate of average step length, squared step length, step time, and cosine of the turning angle for each queen, we calculated the diffusion coefficient for each queen (Turchin, 1998). We compared diffusion coefficients for bees searching for nests within different land cover types using a linear model, after estimates of D were log transformed to better meet assumptions of normality. Second, we made statistical comparisons of movement parameters between land cover types, which required transforming data to better meet assumptions of normality (the dependent variables step length and step time were log-transformed, whereas the dependent variable cosine of the turning angle was logit transformed after rescaling data from 0 to 1). After transforming data, we compared the mean length of each step, cosine of the turning angle, and step time of queens searching for nests in different land cover types statistically using linear mixed effects models with a random effect of flight path ID.

In addition to analyzing movement parameters as outlined by Turchin (1998), we quantified nest-searching behavior of queens by calculating the proportion of time each queen spent inspecting potential nest sites from the ground. We fit a linear model to this data, with land cover type as a predictor variable.

The significance of predictor variables for linear and linear mixed-effects models were determined using F-tests and Wald chisquare tests, respectively, implemented using the Avova function (package car, Fox and Weisberg, 2019). Post-hoc comparisons of estimated marginal means were made using emmeans function (package emmeans, Lenth, 2020).

Results

We recorded the flight paths of 95 queen bumble bees (*B. impatiens*) searching for nests sites at Appleton farms. Of these, 59 individuals inspected at least 3 potential nest sites from the ground, with four individuals searching for nest sites in several land cover types during a single flight path.

We calculated a diffusion rate of 0.366 m^2/s for queens searching for nests in hay fields, 0.119 m^2/s for queens in meadows, and 0.224 m^2/s for queens in forests (Figure 3.3). High diffusion in hay fields is primarily attributable to differences in step length across land cover types (χ^2 = 7.603, df = 2, P = 0.022): queen *B. impatiens* had longer step lengths when searching for nest sites in hayfields (3.3 m) than in meadows (1.8 m) and forests (2.1 m, Figure 3.4a). Turning angles did not differ significantly across land cover types (χ^2 = 3.720, df = 2, P = 0.156), however, the rank-order of the estimates do align with expectations: sharper turns were made by queens searching for nests in meadows (50.8°) and forests (58.8°) when compared to queens searching for nests in hayfields (41.2°, Figure 3.4b). Diffusion coefficients for queens searching for nests in meadows were lower than for queens searching for nests in forests (Figure 3.3), a difference likely attributable to differences in step times across land cover types (χ^2 = 7.603, df = 2, *P* = 0.022). Step times were shorter for queens searching for nests in forests (16.5 s) relative to meadows (32.9 s, Figure 3.4c), though queens searching for nests in hay fields had longer step times (43.7 s) than queens searching in both forests and meadows (Figure 3.4c).

The proportion of time queens spent inspecting potential nest sites from the ground than from the air differed across land cover types (*F* = 4.517, df = 2, *P* = 0.015). Nest-searching queens spent a greater proportion searching of time inspecting potential nest sites from the ground than from the air in forests (52%) relative to hayfields (35.8%) and meadows (37.0%, Figure 3.5).

Discussion

Nest-searching *B. impatiens* moved in a way that would decrease their rate of movement in land cover types associated with high nest densities (i.e. forests and meadows) than in the non-habitat (i.e. hayfields, Figure 3.3). Queens stopped more frequently to inspect potential nest sites in meadows and forests than in hayfields, and although there was no significant difference in turning angles of queens searching for nests in different land cover types, the rank-order of estimates matched our expectations (Figure 3.4). Our findings align with the anecdotal observations of Webb (1961), who noted *Bombus* queens seemed to move more directionally where grass was short, e.g. pastures, than when vegetation was relatively undisturbed. More broadly, our results are consistent with the theoretical prediction that animals should move in a way that maximizes time spent in high-quality habitat, as well as many empirical studies of butterfly movement, in which calculated diffusion coefficients are lower in areas where resources are abundant (Crone et al. 2019).

Studies of movement ecology often propose a simple dichotomy between habitat and non-habitat. However, it is clear that animals also respond to differences in habitat structure and resource availability within these broad categorizations (Schultz et al. 2012b, Brown et al. 2017). For example, Baltimore checkerspot butterflies (*Euphydryas phaeton*) tend to disperse more slowly through forested matrix than grassland matrix, likely because lower temperatures in forests inhibit butterfly movement (Brown et al. 2017, Crone et al. 2019). Here, we observed differences in bumble bee movement between the two landcover types defined as broadly as habitat, in that *B. impatiens* queens searching for nests in forests had higher diffusion coefficients than queens searching for nests in meadows (Figure 3.3). The drivers behind these differences remain unclear. Previous research within this study region has shown *B. impatiens* colonies found in forests tend to have higher reproductive output than colonies found in meadows, suggesting forests provide higher-quality nesting habitat (Pugesek and Crone, *in press*). As such, we expected lower diffusion coefficients in forests when compared to meadows. However, we did not qualify the distribution of nesting resources across the landscape – it may be that, though colonies nesting in forests have better outcomes, nesting resources are more abundant in meadows. Alternatively, differences in diffusion coefficients may have been driven by differences in the ability of queens to locate potential nest sites within different land cover types. For other taxa, habitat structure is thought to play an important role in the search tactics used to locate resources (Robinson and Holmes 1982, Holmes and Recher 1986); animals searching in dense vegetation may need to perform more thorough searches, and thus move at slower rates, to locate cryptic resources. Although the cues used by bumble bees to locate nest sites are not well understood, some species of nest-searching queens seem to be attracted the entrances of rodent burrows and fallen logs (Lanterman et al. 2019), resources that could be easily obscured by vegetation or leaf litter. Given that other authors have raised concerns that the abundance of nest-searching queens is more indicative of inability to locate a suitable nest site than preference (O'Connor et al. 2017), evaluating whether vegetation structure impacts the ability of queens to locate nest sites would certainly be a valuable area of future research.

Researchers often account for movements during multiple behavioral modes (e.g., foraging, resting) when estimating diffusion coefficients of target species (Root and Kareiva 1984, Evans et al. 2020). However, during our surveys, we were unable to account for movement of queens when engaged in non-nest-searching behaviors, as queens often moved too quickly to follow. Although we observed differences in the movement behavior of queens in habitat and non-habitat, it is possible that, by only monitoring movements made when queens were engaged in intensive, slow-paced searches for nest sites, we underestimated the magnitude of these differences. For other insect taxa, differences in diffusion coefficients between matrix and habitat can be extreme. For example, diffusion coefficients of female fender's blue butterflies are14 times larger in non-habitat than in areas where host plants are abundant (Schultz, 1998), a disparity that is not unusual for small butterflies (Crone *et al.*, 2019). Here, in contrast, the average diffusion coefficient of nest-searching queen bumblebees was only 1.5 larger in hayfields than in forests and 3 times larger in hayfields than in meadows. In the future, this limitation could be partially resolved using small radio-trackers. While trackers are too heavy to use on most insects species, a few studies have successfully used ratio telemetry or harmonic radar to monitor the flight paths of large-bodied bees like *Bombus* and *Exaerete frontalis*, typically with the objective of exploring habitat use or estimating home range sizes (Wikelski et al. 2010, Hagen et al. 2011, Makinson et al. 2019). Although radio-trackers weigh down bees and have an adverse effect on fitness (Hagen et al. 2011), flight path data collected from queens using radio tags could be particularly valuable in identifying barriers to queen movement and in identifying area-restricted searches of queen bumble bees at varying spatial scales.

Though there are some limitations to conducting on-the-ground surveys of queen bumble bee movement, our methods offer several advantages over transect counts in evaluating *Bombus* nesting habitat. First, by quantifying animal movement, we gain a more mechanistic understanding of patterns of abundance (Bélanger and Rodríguez 2002). Thus, movement-based metrics of habitat quality may be more reliable than estimates of abundance, especially if nest-searching queen abundance is more indicative of the number of queens that have not yet found suitable nest sites than habitat preference (O'Connor *et al.*, 2017). Second, habitat structure and/or the behavior of queen bumble bees while searching for nest sites may impact the ability of researchers to detect queens. In the spring, queen bumble bees have been observed to spend most of their time on the ground, often burrowing beneath leaves or vegetation (Webb 1961, Makinson et al. 2019). At our study site, nest-searching queens spent a greater proportion of time searching for nests from the ground in forests than in other land cover types (Figure 3.5). As bees under leaf litter or hidden in dense grass are probably less conspicuous than bees in flight, our results suggest that detectability of queens may differ across land cover types. Third, monitoring the abundance of nest-searching queens may not be feasible in some areas. For example, in 2019, our lab group surveyed 9 sites in Napa Valley for a 4-week period, encountering only 8 nest-searching queens during transect surveys, a sample size insufficient to make statistical comparisons (N. Rosenberger, G. Pugesek, N. Williams, and E. Crone, unpublished data). In other studies of nest searching queen abundance, rare species may represented by only 1 or 2 sightings (Svensson et al. 2000; Lanterman et al. 2019). In contrast, useful data can be gleaned from monitoring the movement of ~ 10 individuals, as long as flight paths are long enough (Lode 2000, Byrne et al. 2014).

For several other insect taxa, the relationship between habitat quality and movement has been studied extensively (Crone et al. 2019). To our knowledge, no study has leveraged a similar approach for nesting Hymenoptera (bees, ants, and wasps), at least until now. Using traditional models of animal movement, we found quantifying flight paths of nest-searching queen *B. impatiens* to be a promising method for identifying bumble bee nesting habitat. Our methods required minimal manipulation of individuals (i.e. we did not fit queens with radio trackers) and allowed targeted monitoring of a single bumble bee species. Although the species targeted in this study, *B. impatiens*, is a relatively common bumble bee species, we are hopeful that these methods will be applicable in other study systems, especially for bumble bee species of conservation concern.

Acknowledgements

Funding for this research was provided by the Tufts University College of Arts and Sciences. We would like to acknowledge Risa McNellis for her assistance in the field and Russell Hopping for his assistance in locating field sites. Lastly, we thank The Trustees for their support.

Figures

Figure 3.1. A bumble bee flight path with two steps. Points A, B, and C represent locations where the focal individual inspected a potential nest site from the ground. The turning angle between the two steps is represented by the letter theta (Ɵ).

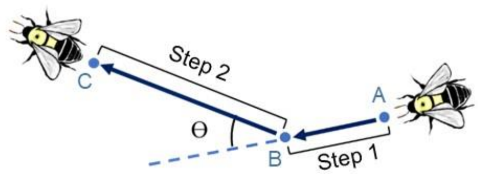


Figure 3.2. Example flight paths of queen *B. impatiens* searching for nest sites in a. hayfields, b. forests, and c. meadows.

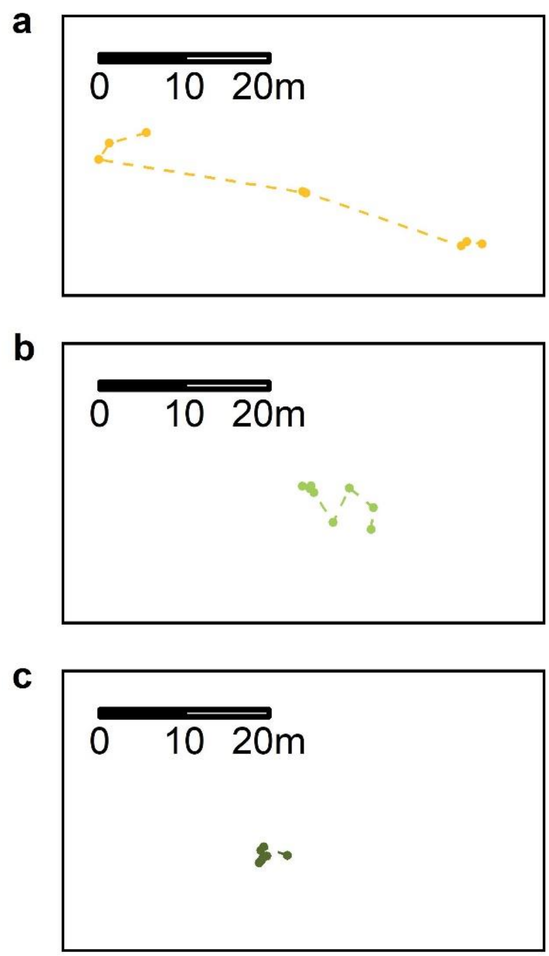


Figure 3.3. The diffusion coefficient, *D*, for queen bumble bees searching for nests within hay fields, forests, and meadows calculated using the correlated random walk approach. Asterisks indicate significant pairwise comparisons, with + representing p-values between 0.1 and 0.05, * representing p-values between 0.01 and 0.05, ** representing p-values between 0.001 and 0.01, and *** representing p-values less than 0.001.

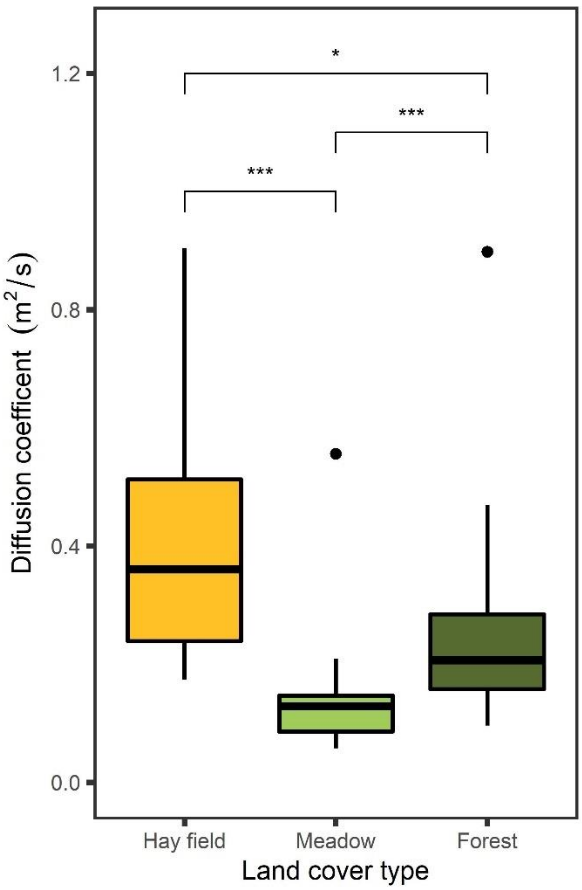


Figure 3.4a. Average step distance, **2.4b.** average turning angle, and **2.4c.** step time for queen *B. impatiens* searching for nests in three land cover types. Error bars represent 95% confidence intervals. Asterisks indicate significant pairwise comparisons, with + representing p-values between 0.1 and 0.05, * representing p-values between 0.01 and 0.05, ** representing p-values between 0.001 and 0.01, and *** representing p-values less than 0.001.

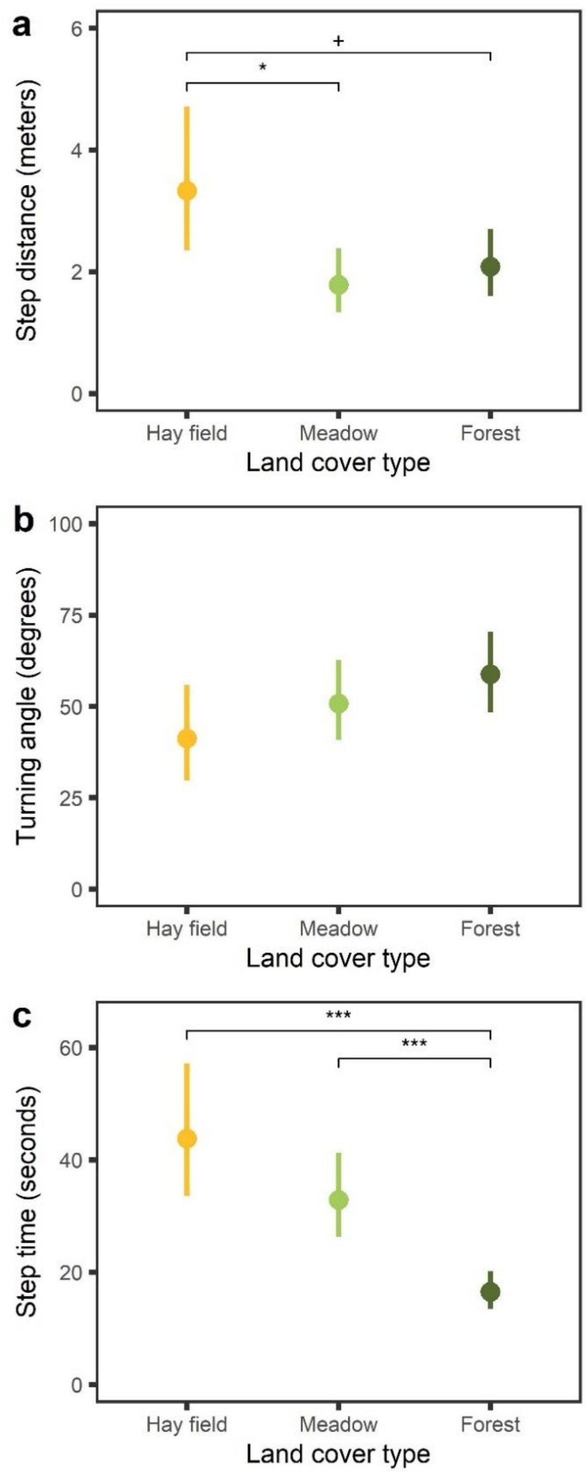
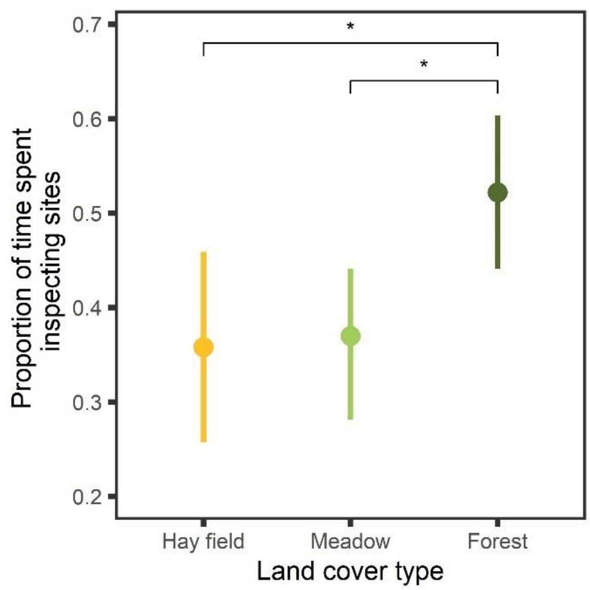

a
+
*
6
4
2
0
Step distance (meters)
Hay field
Meadow
Forest
Land cover type
b
100
75
50
25
0
Turning angle (degrees)
Hay field
Meadow
Forest
Land cover type
c
***
***
60
40
20
0
Step time (seconds)
Hay field
Meadow
Forest
Land cover type

Figure 3.5. The proportion of time spent by queens searching for nests on the ground as compared to in flight. Error bars represent 95% confidence intervals. Asterisks indicate significant pairwise comparisons, with + representing p-values between 0.1 and 0.05, * representing p-values between 0.01 and 0.05, ** representing p-values between 0.001 and 0.01, and *** representing p-values less than 0.001.



Chapter 4:
The effects of commercial propagation on bumble bee (*Bombus impatiens*) foraging and worker body size.

Accepted to *Apidologie*:

Genevieve Pugesek, Carolyn Burtt, and Elizabeth E. Crone (2021) The effects of commercial propagation on bumble bee (Bombus impatiens) foraging and worker body size.

Abstract

1. Bumble bees (*Bombus* spp.) have been commercially propagated for over three decades. As the environmental conditions experienced by commercial bumble bees differ greatly from those experienced by wild bumble bees, commercial rearing of bumble bees may cause phenotypic changes.
2. Here, we compare the foraging behavior and size of worker bumble bees (*B. impatiens*) from commercial and wild colonies. For this experiment, we measured worker body size, recorded and if the workers returned with pollen, and examined the contents of pollen loads via microscopy.
3. We found that, while commercial and wild bumble bees foraged on similar communities of flowers, wild bumble bees returned to colonies with purer pollen baskets (higher proportion of the most common species) and were more likely to return to the colony with pollen than their commercial counterparts. Commercial bumble bees were also smaller than wild bees.
4. Our work highlights differences between commercial and wild bumble bees, in addition to raising important unanswered questions about the mechanism and drivers of these differences.

Introduction

The use of commercially propagated animals as study subjects is common in biological research. Though these animals are often assumed to represent their wild counterparts, the environmental conditions and selective pressures experienced by commercially propagated animals may differ from those experienced by animals in the wild. Differentiation between domesticated and wild organisms has been well characterized across a wide range of taxa, including plants (Harlan 1973), mammals (Trut et al. 2009), and fish (Huntingford 2004), though relatively few studies have addressed the impacts of domestication and commercial rearing on insects (Lecocq 2019). Nevertheless, many species of commercially propagated insects play important roles as scientific subjects: for example, the availability of commercially propagated mason bees (*Osmia* spp.), honey bees (*Apis mellifera*), and several species of bumble bees (e.g, *Bombus terrestris* and *B. impatiens*) has facilitated studies of both social and solitary bees (Pitts-singer and Cane 2011).

The common eastern bumble bee (*B. impatiens*), a social pollinator native to the Eastern United States, has been propagated commercially since 1990 (Velthuis and van Doorn 2006). Though bumble bees have a relatively short history of captive rearing (Velthuis and van Doorn 2006), phenotypic changes in domestic or managed populations are often induced in as few as five generations of captive breeding for other taxa (e.g. hatchery fish, Araki et al. 2008). Commercial bumble bee populations might experience limited gene flow from wild populations, as the producers of commercial bumble bees maintain their own source populations (Velthuis and van Doorn 2006). Artificial conditions are likely to be stressful for bumble bees, as rates of colony success can vary greatly according to rearing techniques (Gurel and Gosterit 2008; Imran et al. 2016) and more than half of wild-caught queens may fail to establish colonies in laboratory conditions (Strange 2010). This stress may lead to selection for performance in lab conditions: previous research has shown commercial *B. terrestris* to produce significantly more gynes and workers than a native genotype in a common garden laboratory rearing experiment, with hybridization between commercial and native genotypes generally resulting in intermediate expression of these colony traits (Gosterit and Baskar 2016). This difference may be unsurprising, given producers of commercial bumble bees are known to select for colonies that establish quickly and grow rapidly (Yoon et al. 2011).

Phenotypic differentiation between commercial and wild organisms could also originate solely from differences in rearing environment. For example, many research animals are fed *ad lib*, which is suspected to lead to the expression of gyne-like characteristics in workers, at least for the social insect *Polistes* (Jandt et al. 2015). Bumble bees reared in artificial conditions may have no experience handling flowers and are typically fed *Apis*-collected pollen (Velthuis and van Doorn 2006), which is lower in protein than pollen collected by bumble bees (Leonhardt and Blüthgen 2012). In addition, producers of commercial bumble bees can manipulate the phenology of colonies by altering the hibernation period of queens (Velthuis and van Doorn 2006). This flexibility allows commercial greenhouse growers to deploy large bumble bee colonies in the early spring, when wild bumble bee workers are not yet abundant. During field studies, it is not unheard of for researchers to put out large colonies (approximately 100 workers strong) as early as May, when *B. impatiens* queens are typically still looking for nest sites (Drummond 2016; Gervais et al. 2020), or to put out similarly sized colonies at different times of the year during a single experiment (e.g., Osborne et al. 1999). Furthermore, although natural *B. impatiens* and *B. terrestris* nest sites are typically found below ground (Plath et al. 1922), commercial colony nest boxes are often installed above the ground to prevent nests from flooding (Osborne et al. 1999, Westphal et al. 2006), and are likely less well insulated from daytime temperatures. These, and other common practices associated with the use of commercial bumble bees in research, have unknown impacts on the behavior of workers once colonies are placed outdoors.

In this paper, we investigate whether inference from commercially propagated colonies of *B. impatiens* differs from inference from wild colonies. Specifically, we present a field experiment designed to compare size and foraging behavior of wild *B. impatiens* workers and workers from commercial colonies installed in the field. As metrics of behavior, we monitored the proportion of workers returning to the nest with pollen and identified the contents of pollen loads to morphospecies. Identifying pollen grains to morphospecies allowed us to assess the purity of each pollen load (i.e. the proportion of pollen grains representing the most common morphospecies in the pollen load). We also measured the intertegular span (IT span) of captured workers as they returned to the nest. Behavioral or morphological differences between bees from wild and commercial colonies could reflect a range of causes, including differences in colony phenology, age and demographic history (e.g., methods of establishing colonies in the lab), as well as possible behavioral and genetic differences due to propagation methods *per se*. In our experiment, including these differences was necessary because we want to represent commercial colonies as they are typically used, and at least some aspects of propagation are proprietary (trade secrets). Thus, if we conclude that bees from commercial colonies are similar to bees from wild colonies, then we can conclude generally that commercially propagated bumble bees are good surrogates for their wild counterparts (at least for the traits we measured). If we find differences, our work could provide motivation for further research into the mechanisms behind these differences.

Methods

Study site

Appleton Farms (~1000 acres) is located in Ipswich, Massachusetts, and is maintained by the Trustees of Reservations. We selected a 0.3 Ha meadow as our study site because of its relatively high worker bee density (Pugesek, personal observation). This meadow is mowed annually in the late fall to prevent succession.

Pollen Collection

We collected pollen from 8 commercial and 8 wild *B. impatiens* colonies over the course of the study period. During each year of the study (2017 and 2018), four commercial colonies (Natupol Excel Observation, Koppert Biological Systems) were installed at the edges of the study site (see Appendix 4.1). Colonies were installed in July; each colony was placed on a wooden pallet and shaded with a tarp tent, in accordance with the recommendations of the supplier to provide shade on all sides of the colony. Colonies were fed sugar water ('Bee-Happy', Koppert Biological Systems) for a period of 1 week, after which we removed the nectar reservoir (included with the artificial nest box) to minimize the effects of feeding (Martin et al. 2018). Colonies were then left to acclimate for an additional two weeks, an acclimation period similar or greater than that of other studies that use commercial bees as study subjects (Osborne et al. 2008a; Couvillon et al. 2010). By the time we began collecting data, we expected high turnover of foraging workers, as their life span in the wild is short (< 20 days in a field study of an ecologically similar species, *B. vosnesneskii*; Kerr et al. 2019). During this three-week acclimation period, we located four wild *B. impatiens* colonies during each year of the study by freely searching the study site for workers entering or exiting colony entrances (Iles et al. 2019). Although commercial colonies were located at the edge of our study meadow (see Appendix 4.1), the field we worked in was quite small, less than half a hectare.

After the acclimation period was complete, workers were netted as they returned to their nest site for about one week. To induce cold anesthesia, workers were placed in vials on ice for 5-10 minutes. For each captured individual we checked for the presence or absence of pollen loads and measured the (IT) span, the shortest distance between the two wing tegulae, a common measure of bumble bee body size (Greenleaf et al. 2007). In 2017, we also collected pollen loads from workers nondestructively using a sterilized razor blade and preserved pollen loads on ice.

After measurements were complete, workers were marked using a sharpie paint pen and released. Marked workers were not repeatedly measured. All workers were collected between 8 am and 3 pm, and we alternated collecting workers from wild and domestic colonies, to ensure that time of day did not bias sampling (Free 1955; Peat et al. 2005).

Pollen identification

Pollen loads were suspended in 100µl ethanol, and 20µl of this solution was combined with 40µl fuchsin gel (Beattie 1971) and plated onto microscope slides. A ZIEISS Axio Scope.A1 microscope was used to view pollen grains, and pollen grains were imaged and measured using Spot 5.2 software. Pollen grains were categorized to one of 13 morphospecies, differentiated by size, surface markings, and shape, though they were not assigned to specific plant species or genera (Appendix 4.2). For each slide, 200 pollen grains were identified to morphospecies, with 40 pollen grains were sampled haphazardly from each corner and an additional 40 sampled from the center of the slide.

Statistical methods

All statistical analyses were performed in R, version 4.0.2 (R Core Team 2019). We compared the body size of workers originating from commercial and wild bees using a linear mixed effects model (lme4), derived using restricted maximum likelihood (REML = TRUE). Bee origin (commercial or wild) and year (2017 or 2018) were included as fixed effects and colony ID was included as a random effect (Bates et al. 2015). (Note that comparison of body size could only be done with foraging workers because *B. impatiens* colonies are subterranean and cannot be nondestructively monitored). Similarity, pollen load presence or absence was analyzed using binomial family, generalized linear mixed effects models (lme4; Bates et al. 2015) with bee origin and year as fixed effects and colony ID as a random effect. The significance of each fixed term was determined using Type II marginal hypothesis tests implemented with the Anova function in the R package car (Fox and Weisberg 2019).

To evaluate the purity of pollen loads, we determined the most common pollen morphospecies contained in each pollen load and calculated the proportion of pollen grains representing this morphospecies from the total sample. We used linear models to analyze data with bee origin as a fixed effect. To meet assumptions of normality, data were logit transformed after rescaling from 0.025 to 0.0975. The significance of bee origin was determined using Type II marginal hypothesis tests implemented with the Anova function.

We compared the morphospecies composition of pollen loads collected by commercial and wild bumble bees using a permutational multivariate analysis of variance (PERMANOVA) with bumble bee origin as a predictor variable, implemented with the function adonis2 (package vegan, Anderson 2017) in R, using Bray-Curtis dissimilarity indices. Data were square root transformed to minimize the effects of most abundant groups. We performed an additional, unconstrained analysis (non-metric multidimensional scaling ordination, i.e., NMDS) to visualize pollen morphospecies abundance data. NMDS was performed using a 2-dimensional solution (stress = 0.1015) and Bray-Curtis dissimilarity indices (function metaMDS, package vegan). Data were square root transformed prior to analysis (once again, to minimize the effects of most abundant groups). The plot produced from NMDS is not meant to represent the results of our constrained analyses (PERMANOVA), as approaches for dimensionality reduction differ between constrained and unconstrained analyses (Paliy and Shankar 2016, Scott and Crone 2020). Our NMDS analysis simply serves to visualize data in a reduced dimensional space.

Results

Over the course of two years, a total of 271 workers were sampled from 16 colonies (4 colonies/treatment/year): in 2017, we collected commercial 102 workers and 101 wild workers, while in 2018, we collected 29 commercial workers and 39 wild workers.

Commercial worker bumble bees were smaller than their wild counterparts (χ^2= 10.618, df = 1, P = 0.001, Figure 4.1). In 2017, average IT span was 3.30 (SE = 0.06) mm for commercial and 3.43 (SE = 0.06) mm for wild workers, while in 2018, average IT span was 3.45 (SE = 0.10) mm for commercial and 3.92 (SE = 0.09) mm for wild workers. Worker bumble bees collected in 2017 were also significantly smaller than bees collected in 2018 (χ^2= 18.705, df = 1, P < 0.001, Figure 4.1). There was a significant interaction between these main effects (χ^2= 4.679, df = 1, P = 0.031): the size difference between commercial and domestic bees was larger in 2018 (Figure 4.1). Worker body size did not differ much among colonies within groups, as indicated by random effect standard deviation of 0.06 for IT span.

Workers from wild colonies were more likely to return to the colony with pollen than workers from commercial colonies (χ^2= 6.72, *df* = 1, *P* = 0.010, Figure 4.2). In 2017, 34.4% (SE = 7.1) of commercial workers and 49.8% (SE = 7.7) of wild workers returned to the nest with pollen, while in 2018, 22.0% (SE = 9.0) of commercial workers and 58.9% (SE = 9.9) of wild workers returned with pollen. There was no significant effect of year on the proportion of workers found returning to the colony with pollen (χ^2= 0.010, *df* = 1, *P* = 0.922), nor was there a significant interaction (χ^2= 1.544, *df* = 1, *P* = 0.214). Pollen foraging differed moderately among colonies within groups, as indicated by random effect standard deviation of 0.46 for the proportion carrying pollen (note that this SD is on a logit-scale)

According to PERMANOVA, the composition of flower morphospecies communities visited by commercial and wild bumble bees did not differ ($F_{1,70}$= 1.5691, R^2 = 0.02192, P = 0.193). In addition, NMDS analysis showed no clear separation of contents of pollen loads collected by commercial and wild bees (Fig. 3). Both commercial and wild bumble bees tended to visit more than one flower morphospecies during foraging bouts: 71 of 72 of pollen loads contained pollen from more than one morphospecies. However, the pollen loads collected by commercial bumble bees were not as pure than those collected wild bees ($F_{1,70}$= 9.317, P = 0.003, Fig 4). Together, these results suggest that while commercial and wild colonies collected the same diversity of pollen, individual workers were more specialized in wild colonies.

Discussion

Workers from commercial and wild bumble bee colonies differ in terms of foraging behavior. While commercial and wild workers visited similar communities of flowers (Figure 4.3), commercial workers were less likely to return to the colony with pollen. Our results also suggest wild bumble bees may be more specialized foragers relative to commercial bees, as wild bees collected purer pollen loads. Past studies have shown that domesticated insects may be as effective as their wild counterparts when handling food, at least in highly controlled laboratory environments (Cohen 2000; Hagler 2009). However, in other taxa, domestication often leads to changes in foraging behavior. For example, domestic pigs (*Sus scrofa*) and fowl (*Gallus gallus domesticus*) move less during foraging (Gustafsson et al. 1999; Andersson et al. 2001; Schütz et al. 2001), suggesting that domestic animals are not as willing to allocate resources to highly energetic foraging behaviors. In contrast, brown trout (*Salmo trutta*) reared in hatcheries tend to move more and feed less while foraging than wild brown trout (Bachman 1984). Domestication can clearly affect foraging behavior of many taxa, including *B. impatiens*.

Foraging workers from commercial colonies were smaller than those of wild origin (Figure 4.1). Our results contrast with those of Ings et al. (2006), who found that foragers from non-native, commercial *B. terrestris* colonies tended to be larger than foragers from colonies of native sub-species. One reason for this difference may be that Ings et al. (2006) compared a native sub-species of *B. terrestris*, *B. t. audax*, to a commercially available non-native subspecies, *B. t. dalmatinus*. Thus, differences in body size observed by Ings et al (2006) may have been driven by differences between sub-species, as opposed to commercialization. In contrast, there are no recognized subspecies of *B. impatiens*. In other insect taxa, artificial selection may lead to larger body sizes (e.g., silkworms, Lecocq 2019), though captive rearing has led to decreased body size for some species of insects (Cohen 2000; Schultz et al. 2009). In the case of bumble bees, it is possible that selection for larger colony sizes (i.e. colonies with more workers) has led to an inadvertent selective pressure for smaller workers. Among larger colonies of *B. impatiens*, workers tend to be smaller (Couvillon et al. 2010b). Production of larger workers requires more resources, and thus there are trade-offs between producing larger workers and producing a larger number of workers for bumble bees (Kerr et al. 2019).

In our study, another cause of variation in body size could be the developmental age or stage of bees in domesticated vs. wild colonies (Chole et al. 2019). Colonies purchased from Koppert (and other commercial suppliers) are classified by the number of workers, rather than colony age or developmental history. Therefore, we did not know the demographic composition of these colonies. We were also unable to infer the age or developmental state of wild colonies, as we located established colonies in July, when colonies were large and more conspicuous. For other bumble bee species, anecdotal evidence has suggested bumble bee worker body size increases as the colony develops (Chole et al. 2019). However, to our knowledge, only one study (Couvillon et al. 2010b) has addressed ontogeny of worker body size distribution in *B. impatiens* colonies, finding no evidence average worker body size changes through time. It is worth noting that the Couvillon et al. study took place entirely in the laboratory – it is possible other patterns could arise in the field. Understanding the mechanisms of body size differences would require some combination of field studies done in close collaboration with commercial suppliers and common garden studies with colonies founded by mated queens either from the wild or from a commercial producer (e.g., Gosterit and Baskar 2016). Nonetheless, colonies of unknown demographic history are now routinely used in ecological research to represent wild bumble bees (Drummond 2016; Gervais et al. 2020). Our study suggests that further research into the differences between commercial and wild colonies would help us understand how to interpret these studies.

Similarly, many factors could contribute to the differences we measured in foraging behavior of workers from commercial and wild colonies. Since our goal was to compare inference from commercial colonies as they are typically used, we installed them as directed by the manufacturer, rather than in a way that was comparable to the conditions experienced by wild bumble bees. *Bombus impatiens* colonies usually occur underground (Pugesek and Crone *in press*); in 2017 we excavated two of these nests at our field site and they were located at the end of several feet of underground tunnels (presumably in abandoned small mammal burrows). These conditions are not feasible to recreate for colonies in nest boxes; instead, Koppert and other commercial suppliers recommend placing colonies above ground beneath trees or under tents to prevent colonies from overheating. Use of above-ground boxes is a common technique to study bumble bees in the field, even with colonies propagated from wild-caught queens (Kerr et al. 2019; Malfi et al. 2019). If these differences in colony position affect foraging behavior of bees in our study, they would also lead to similar confounding factors in many studies that use bees in above-ground boxes to represent wild bees.

Although commercial colonies were installed under trees toward the edges of the field site, we suspect that colony location was not a significant factor in the differences we observed because all colonies were located in a small (< 1 ha) field (Appendix 4.1). Wild colonies were often within 50 meters of commercial colonies. To our knowledge, there are no published estimates of *B. impatiens* foraging ranges; however, other species of bumble bees readily forage more than 250 meters from nest sites (Osborne et al. 1999, Walther-Hellwig and Frankl 2000), with some workers foraging at distances greater than a kilometer (Osborne et al. 2008a, Walther-Hellwig and Frankl 2000). If *B. impatiens* foraging ranges are similar, we would expect that foraging ranges of commercial and wild bumble bees would overlap almost completely (Appendix 4.1). In addition, we did not observe any differences in the overall pollen composition collected by workers from commercial vs. wild colonies (Figure 4.3), suggesting that they were foraging for similar resources. Rather, the difference was in the purity of loads brought back by single workers.

It is tempting to speculate that the two effects we observed – differences in body size and foraging behavior – are related. In some bumble bee species, larger workers collect nectar at a faster rate than smaller bumble bees (Goulson et al. 2002). In fact, Ings et al. (2006) cites differences in foraging worker body size as a potential driver of differences in nectar return between lab-reared *B. t. audax* and commercial *B. t. dalmatinus*. In that study, commercial bumble bees weighed 20-70% more than wild workers (depending on the study site location), compared to a 9% difference of average IT span (3.35 vs. 3.63 mm) in our study. Kerr et al. (2019) observed essentially no change in the probability that larger *B. vosnesenskii* workers would carry pollen over this size range, although very large workers (4.5 mm IT span) were more likely to forage for pollen, and carried slightly more pollen per trip. In our data, IT span was not a significant predictor of the probability workers would carry pollen, after accounting for colony origin (Appendix 4.3). Based on these results, it may be parsimonious to conclude that differences in body size and pollen foraging result from different causes.

Large workers have a better memory and are faster learners than small workers (Worden et al. 2005; Riveros and Gronenberg 2009). Thus, larger workers may be more suited to challenging or complex foraging tasks like collecting pollen or handling flowers. This result could explain the difference in pollen purity, if we reason that "majoring" on a species during foraging bouts is a complex behavior. However, it could be argued that "majoring" simplifies the foraging process and would require less cognitive capability. Like many aspects of the effects of domestication on bumble bees, the causes and consequences of this difference would be a fascinating direction for future research.

The use of commercial bumble bees, both for pollination services and for research, has increased tremendously since the late 1980's (Velthuis and van Doorn 2006), and there is growing interest in identifying new candidate species for commercialization (Baur et al. 2019). Commercially propagated bumble bees (*B. impatiens* and/or *B. terrestris*) have been used to evaluate a wide range of foraging behaviors: floral preference (Vaudo et al. 2014), worker bumble bee foraging ranges or trip duration (Osborne et al. 1999, 2008a; Westphal et al. 2006), and worker foraging performance or resource return (Goulson et al. 2002; Raine and Chittka 2008; Feltham et al. 2014). Researchers have frequently used commercial colonies to evaluate foraging worker behavioral response to colony manipulation of pollen or nectar stores (Plowright et al. 1999; Kitaoka and Nieh 2009; Hendriksma et al. 2019) or with exposure to insecticides (Feltham et al. 2014). If our results represent commercially propagated bumble bees in general, they inform our inference about which kinds of studies commercial bumble bees would best represent wild bumble bee colonies. For example, inference about pollen foraging (Leonhardt and Blüthgen 2012) and floral preferences of workers (Vaudo et al. 2014; Drummond 2016) may be transferrable from commercial to wild populations, given that we observed commercial and wild bumble bees to visit similar communities of flowers (at least in terms of morphospecies). However, inference about net resource return (Goulson et al. 2002; Feltham et al. 2014), or preferences for pollen over nectar (Plowright et al. 1999; Peat and Goulson 2005; Hendriksma et al. 2019) may be less transferrable, if (as suggested by our results) wild bumble bees generally return with pollen more frequently or are more likely to major on particular species while foraging for pollen. This study highlights that, although commercial bumble bees are similar to wild bumble bees in many ways, they are not perfectly substitutable.

In spite of these differences, we do not seek to completely discourage the use of commercial bumble bee colonies in research. Many of the published studies which use commercial bumble bees as study subjects would not otherwise have been possible, and there are many benefits to using similarly sized bumble bee colonies in experiments. However, we believe it is important to recognize the caveat that the bees used in these experiments may well differ from their wild counterparts. Although challenging, it is possible to locate bumble bee colonies in the field with large enough sample sizes to ask focused ecological questions (cf. Harder 1986, Pugesek and Crone *in press*). In the future, it will be useful to complement research on domesticated bumble bees with research on wild colonies.

Acknowledgements

We thank Annika Greenleaf for her assistance with field work, and the Trustees of Reservations for allowing us access to their properties. A special thanks to Sarah Richman, Jake Francis, and Dr. Anne S. Leonard for providing feedback on this manuscript prior to publication.

Figures

Figure 4.1 The average intertegular (IT) span of foraging worker bumble bees from commercial and wild colonies. In 2017 and 2018, 203 and 68 worker bumble bees were measured, respectively. Error bars represent 95% confidence intervals.

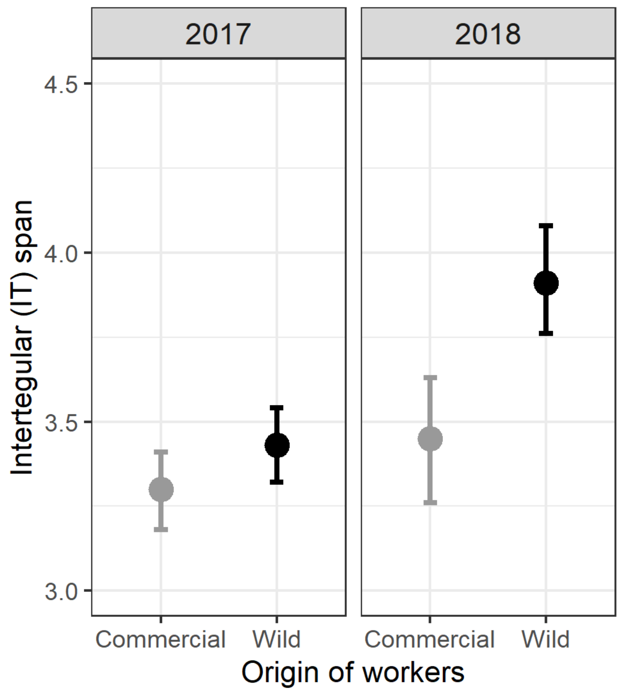


Figure 4.2 The proportion of foraging worker bumble bees found to return with pollen for bumble bees from commercial and wild colonies. In 2017 and 2018, 203 and 68 worker bumble bees were measured, respectively. Error bars represent 95% confidence intervals.

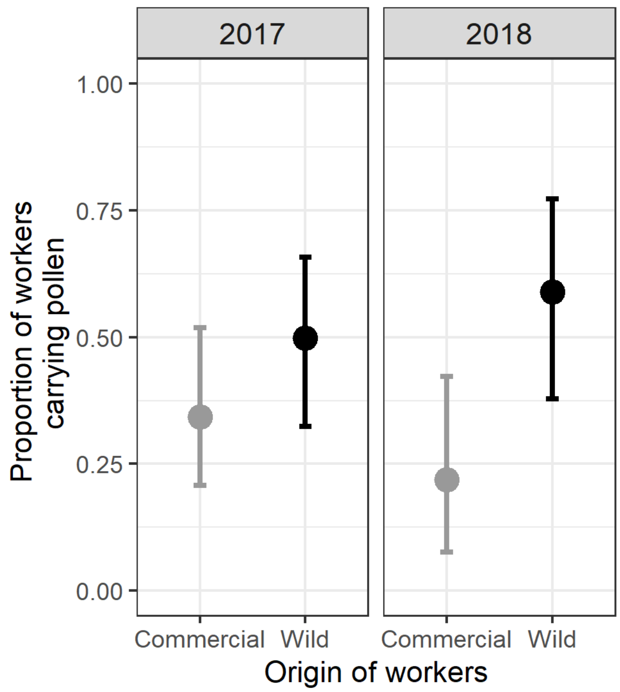


Figure 4.3 Non-metric multidimensional scaling ordination plot of morphospecies composition of pollen loads for commercial and wild bumble bees, with convex hulls drawn to enclose points in each group.

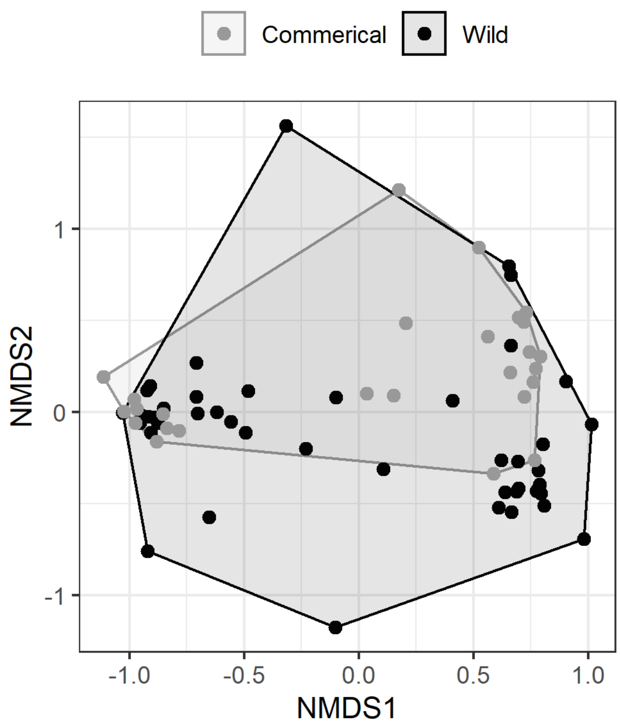
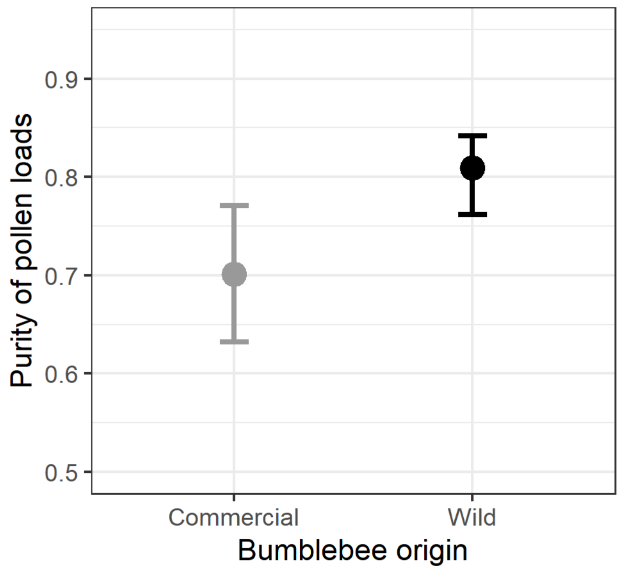


Figure 4.4 The purity of pollen loads (i.e. the proportion of pollen grains representing the most common morphospecies in a pollen load) collected by commercial and wild bumble bees in 2017. Error bars represent 95% confidence intervals.



Appendix 4.1

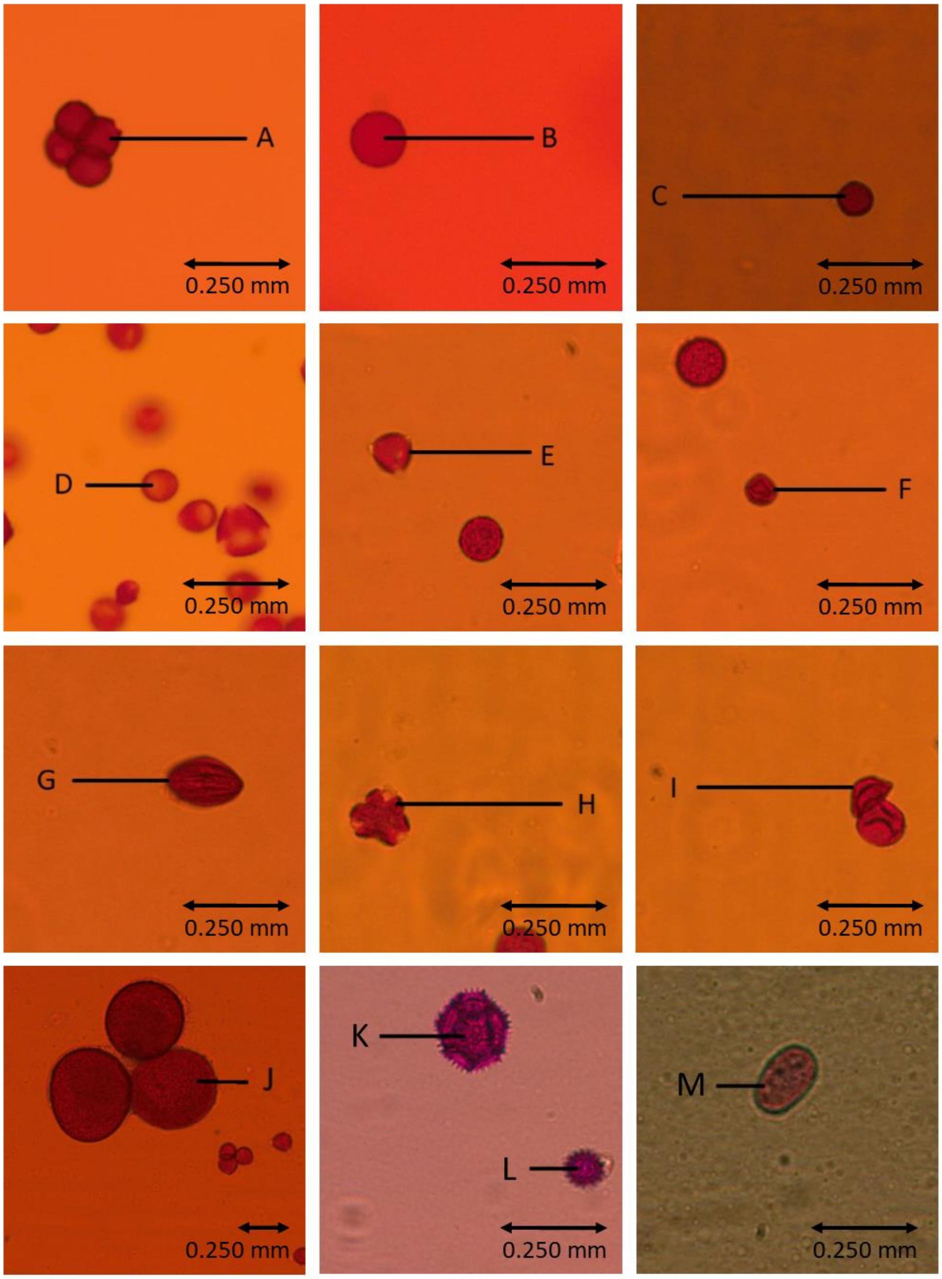
Figure S4.1 The locations of *B. impatiens* colonies at our study site. Wild colonies are marked with a light blue circle, while commercial colonies are marked with a dark blue triangle. Images were created in ArcMap. Data sources include Esri, DigitalGlobe, GeoEye, i-cubed, USDA FSA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community.

Appendix 4.2

Table S3.1 Descriptions of pollen morphospecies identified.

#	Shape	Length (μm)	Description
A	Clumped	NA	3-5 lobes connected at a center point
B	Round, pocked surface	50 – 150	Spherical with approximately 5-6 evenly-spaced light-colored circles on exterior wall
C	Round, circumscribed by line	50 – 100	Spherical with distinct dark ring around circumference
D	Round, small	50 – 100	Spherical with one pole of a lighter color than the other. Occasionally more of a teardrop shape than completely spherical.
E	Tricorn	75 – 175	Tricorn, with three equidistant light bands emanating from center and ending on the outside of the sphere.
F	Crumpled	50 – 100	Spherical with a raisin-like appearance due to uneven lines covering the exterior.
G	Bead-like	150 – 200	Prolate spheroid, with 4 equidistant lengthwise lines on surface. Shaped like a lemon.
H	Giftwrap bow	125 – 200	Similar to a tricorn pollen, but larger and with surface indentations between the lines.
I	Hemisphere	100 – 150	Hemispheric. Two u-shaped lines on the surface with the bottom of the "u's" touching.
J	Round, large	250 – 400	Spherical, with dark line around circumference.
K	Spiky, small	50 – 100	Spherical, with a spikey surface
L	Spikey, medium	150 – 200	Spherical, with a spikey surface
M	Ovaloid	150 – 200	Ovaloid with visible variegated surface color

Figure S4.2 Photographs of each pollen morphospecies identified. Images were captured using Spot 5.2 software.

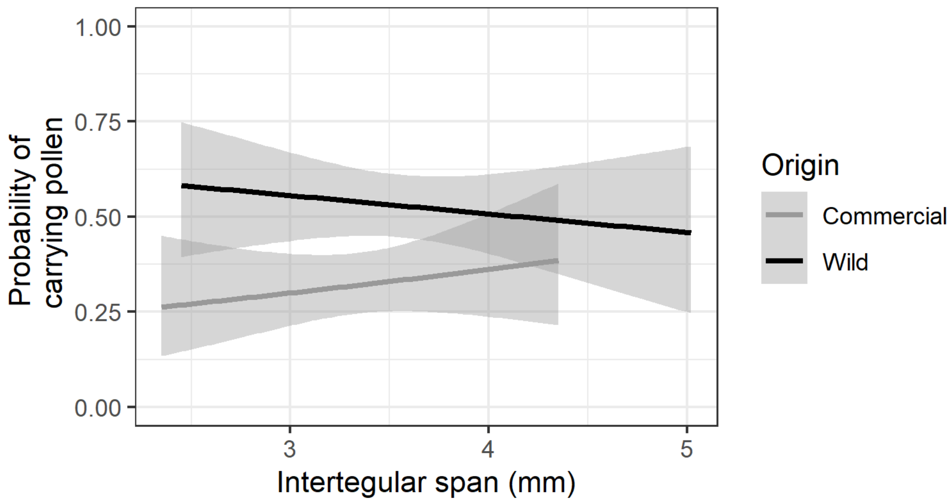


Appendix 4.3

To evaluate potential differences in the probability of carrying pollen for large and small worker bees, we a built single generalized linear model (Binomial family, logit-link) to test for differences in the probability of carrying pollen as a function of bee origin (commercial or wild) and intertegular (IT) span. We also included an interaction term between bee origin and IT span. The significance of each term was determined using Type II marginal hypothesis tests implemented with the Anova function in package car (Fox and Weisberg 2019). All statistical analysis were performed in R, version 4.0.2 (R Core Team 2019).

We found worker bumble bees from wild colonies were more likely to return to the colony with pollen than worker bumble bees from commercial colonies (χ^2= 11.518, *df* = 1, *P* < 0.001, Fig. S1). However, there was no significant effect of IT span on the likelihood of a worker bumble bee to return to the colony with pollen (χ^2 < 0.001, *df* = 1, *P* = 0.994, Fig. S2), nor was there a significant interaction (χ^2= 0.948, *df* = 1, *P* = 0.330, Fig. S2).

Figure S4.3 The effect of intertegular span (ITS) on the probability of returning to the nest with pollen for bumble bees of commercial and wild origin. Shaded regions represent 95% confidence intervals.



Chapter 5:
Diapause survival of queen bumble bees in the field is higher than in past lab studies.

In preparation:

Genevieve Pugesek, Jessie A. Thuma, and Elizabeth E. Crone (2021) Diapause survival of queen bumble bees in the field is higher than in past lab studies.

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Abstract

1. Protecting target species during sensitive life cycle stages is a fundamental goal of conservation ecology. But first, it is necessary to identify at which points of the life cycle populations are most vulnerable. For bumble bees (*Bombus* spp.), an ecologically and economically valuable pollinator taxon, there is some concern that diapause may be a particularly sensitive life cycle stage, given that the success of overwintering queens is critical to next year's bumble bee population, and that queen bumble bees overwintered in the lab often have low survival rates. It is, however, unclear how lab-based estimates of diapause survival compare to survival rates of natural populations.
2. In this study, we monitored the survival of *Bombus impatiens* queens overwintering in the field, and conducted a meta-analysis of studies that estimate queen diapause survival in the lab, to compare our field-based estimates of survival to those of lab-based studies.
3. We found that queen *B. impatiens* had relatively high rates of overwintering survival after ~ 6 months (>60%), especially when compared to estimates of 6-month survival from lab studies (~10%). We also noted a trend that broadly corroborates the majority of lab studies of bumble bees, in that larger queen bumble bees were more likely to survive until spring.
4. In addition to emphasizing the value of relating patterns observed in the lab to observations in nature, our study provides the first estimate of diapause survival for queen *Bombus* in nature.

Introduction

Diapause is a strategy that many species of insects rely upon to cope with adverse environmental conditions (Denlinger 1986, Gill et al. 2017). However, this stage of the life cycle can be both energetically and physiologically taxing (Hahn and Denlinger 2011, Sinclair 2015). Insects in temperate regions, which typically enter diapause to better cope with freezing temperatures, may spend more than 70% of their lifespan in diapause (Gill et al. 2017) There is concern that some insect species may be particularly vulnerable to mortality during diapause, and that low overwintering survival rates may lead to "demographic bottlenecks" (c.f. Treanore and Amsalem 2020), where conditions experienced during the winter disproportionately impact population dynamics. However, identifying how sensitive natural populations are to overwintering mortality can be challenging. Many of the studies that address insect diapause are carried out entirely in the lab, with the goal of characterizing the physiological mechanisms that underpin survival (Leather et al. 1993). Monitoring vital rates in the lab allows researchers to better control sources of environmental variation; however, the conditions experienced by animals in artificial settings are not always ecologically realistic. As such, the extent to which discoveries in the lab can be extrapolated to natural populations is often unclear.

To address this knowledge gap, we conducted a field-based study to estimate diapause survival of queen bumble bees (*Bombus impatiens*) in Ipswich, MA, USA. Bumble bees are one of the most important native pollinators of crops in the United States (Kleijn et al. 2015), partially because bumble bees are one of the few native pollinators that can be reared commercially. In the Northeastern United States, commercial *B. impatiens* colonies are commonly used by farmers to enhance pollination of greenhouse tomatoes (*Solanum lycopersicum*) as well as a number of other crops. Though there is considerable interest in exploring the environmental variables that impact bumble bee diapause survival, most of the studies that address this stage of the life cycle focus on improving methods of husbandry (Beekman et al. 1998, Lindsay 2020). In fact, these researchers may have very little interest in simulating the conditions experienced by bees in the wild (e.g., Beekman et al. 1998). Maintaining queen bumble bees during diapause can pose a significant hurdle in rearing captive populations: many lab studies recommend overwintering queens for only a few months to maximize productivity (Beekman et al. 1998, Gosterit and Gurel 2009). However, in nature, queens typically spend at least 6-9 months in diapause (Alford 1969, Pouvreau 1970). To our knowledge, no study has addressed whether low diapause survival rates are typical in the wild.

To date, locating queen bumble bees in sufficient numbers has posed a major hurdle in studying diapausing bumble bees in the field. Bumble bees overwinter below the ground (Alford 1969), and like most social Hymenoptera, overwinter solitarily (Leather et al. 1993). There are, however, a handful of species of social bees which overwinter in large aggregations around natal nest sites (Plath 1927, Sakagami et al. 1984). *B. impatiens*, the common eastern bumble bee, is the only *Bombus* species reported to overwinter in large groups (Plath 1927). Studying this species thus offers a unique opportunity to monitor large numbers of overwintering queens.

In this study, we estimate diapause survival rates for *B. impatiens* in the field by monitoring queens discovered around nest sites. We also test the relationship between body size and overwintering survival, as queen size has been previously identified as an important driver of overwintering mortality (at least in laboratory settings). To compare our estimates of diapause survival to those produced by lab-based studies, we conducted a meta-analysis of past research, using Web of Science and OATD (Open Access Theses and Dissertations) to build a data set using 16 different studies. In addition to providing general information regarding the locations of overwintering *B. impatiens* queens, our study is the first to estimate overwintering mortality of natural bumble bee populations (but see Szabo and Pengelly 1973 for the most closely related study).

Methods

Study site

All field work was performed at Appleton Farms (42°38'52.09"N, 70°51'1.01"W) and Appleton Grassrides (42°38'33.26"N, 70°51'57.12"W), two adjacent properties located outside of Ipswich, MA, USA. Both properties are owned and maintained by the Trustees of Reservations, a non-profit land stewardship organization.

Locating overwintering queens.

In the fall of 2019 and 2020, we performed systematic searches around the entrances of *B. impatiens* nests to look for queens actively digging overwintering sites, or for patches of soil disturbed by queens (Figure 5.1A and B). Over the course of the study, we searched for evidence of overwintering queens around 4 *B. impatiens* nest sites (3 of these nests were located in 2019, during other field work [Pugesek and Crone, *in press*]), whereas the last nest site was located in 2020, during a meandering search for *Bombus* nests at Appleton Farms and Grassrides. We only searched for overwintering queens around nest sites located in forests (during other field work, we observed queens excavating hibernacula around nest entrances in forests and open grasslands; however, we avoided searching around nest sites in grasslands, because it was difficult to find evidence of hibernacula under dense grassland vegetation).

From the first week of September until the end of October, we visited nest sites once per week to mark the locations of overwintering queens. During each visit, we searched 7-9 quadrats (each 16 m^2) around each nest site for evidence of *B. impatiens* hibernacula (Figure 5.1A). Quadrats were laid out at varying distances from nest site entrances (with the furthest 10 meters from the colony entrance, though, on average, overwintering sites were found between 1.5-2 meters from nest entrances [see Table 5.1]). The same quadrats were searched each week for approximately 10 minutes per visit. During each search, we marked potential overwintering sites with uniquely numbered, aluminum tags.

Recovering overwintering queens

To confirm the presence of overwintering queens in the soil, we excavated 141 marked overwintering sites in late November and early December (approximately 35 overwintering sites, selected randomly, were excavated from each of the four aggregations [Table 5.1]). To excavate each queen, we gently dug around the marked entrance of the overwintering site. Many queens were found just a few centimeters below the surface, in distinct hibernacula. However, if queens were not immediately located, we dug approximately 10 cm below the soil surface before ceasing our search.

Differentiating between live and dead queens was fairly straightforward. Dead queens were often partially decayed or covered in mold, whereas live queens had pristine coats and became active upon disturbance (see Figure 5.1C). We measured the depth at which each queen had been buried, and gently dislodged queens from their overwintering positions using either a bristled brush or a digging utensil. Live queens were immediately placed on ice to prevent complete disruption of hibernation. We measured the intertegular span of all live queens, and marked each queen with a uniquely numbered, fluorescent plastic tag (queen bee marking kit, Better Bee). We then reburied queens in their original positions and replaced the numbered metal plant tag in the soil above each queen. We re-excavated queens in late March (March 24th, 2020 and March 21st, 2021). We used similar methods to determine whether queens had survived over the winter, though, in the spring, dead queens were far more degraded than they had been in the fall (see Figure 5.1D).

As queens began overwintering sometime between August and mid-October, there was some variability in the amount of time queens had spent overwintering. However, most queens had been overwintering for approximately 2 months when they were first checked, and 6 months when they were checked for the second time.

Statistical Analysis (Field Data)

We used generalized linear models (binomial family, logit link) to estimate survival rates for queens monitored at Appleton Farms and Grassrides (all statistical analyses were performed in R, version 4.0.2 [R Core Team 2019]; GLMS were run using the command glm(), one of the basic functions in R). Separate sets of models were run to estimate survival at two different time points: after ~ 2 months, and after ~ 6 months. At 2 months, the response variable was coded based on whether queens were found live (=1) or dead (=0) (if no queen was recovered when the overwintering site was excavated, we assumed that the site had been abandoned, as, according to the observations of Alford [1969], queens may desert burrows if an obstruction is encountered while digging). At 6 months, the response variable was coded two ways. First, we coded the response variable as whether or not the queen was found live in the spring (= 1) or dead either during the fall or the spring (= 0). Second, as a more cautious estimate of survival after 6 months, we also ran a set of models where the response variable was coded as 1 if found live in spring, or as 0 if the queen was found dead in either the spring or fall or had not been recovered at all in the spring.

Our study was limited, in that only 4 aggregations of overwintering queens were monitored. Thus, to estimate survival at each time point, we ran two separate models. First, we pooled data across aggregations, and estimated the proportion of queens to survive using an intercept-only model. Second, we ran a model with aggregation ID included as a predictor variable, to determine whether survival rates differed between aggregations. The significance of fixed terms (i.e. aggregation ID) was determined using Wald chi-square tests implemented with the Anova() function in the package car (Fox and Weisberg 2019).

To examine the effects of IT span (measured in the fall, when queens were first excavated) on survival (measured in the spring), we used multinomial logistic regression models. Although survival is typically regarded as a binomial variable, we categorized the response variable into three groups: live, dead, and not recovered. Due to low sample sizes, we ran three separate models to explore the relationships between survival, IT span, and aggregation ID. First, we pooled data across aggregations, and ran a model that included only IT span as a predictor. Second, we ran a model that included only aggregation ID as a predictor. Third, we ran a model that included additive effects of both aggregation ID and IT span as predictors. Multinomial models were fit using the function multinom() in R package nnet (Venables and Ripley 2002). The significance of fixed terms (i.e. aggregation ID) was determined using Wald chi-square tests (type II) implemented with the Anova function.

Data Extraction and Meta-analysis

To find estimates of *Bombus* overwintering survival, we conducted a systematic literature search on April 12th, 2021, using Web of Science and Open Access Theses and Dissertations. A total of 38 research articles and 2 theses/dissertations were obtained using the search terms: (Bombus OR "bumble bee" OR bumblebee) AND (overwinter* OR diapause*) AND (survival). We excluded studies that did not provide direct estimates of *Bombus* overwintering survival, retaining 19 studies (17 research papers, 1 dissertation, and 1 thesis). A few authors presented the same data sets in multiple publications: 2 additional studies were excluded as duplicate data. A final study was excluded, because queen survival rates could not be calculated from the data as presented. As such, a total of 16 studies were included in our literature review.

Almost all of the studies obtained tested several different diapause regimes (e.g., different temperatures, levels of humidity, etc.), and reported queen survival rates at monthly intervals. Thus, for each set of experimental conditions reported by each study, we recorded the 1) the proportion of queens to survive, 2) the sample size, 3) the length of the diapause regime, 4) the species, mating status, origin (lab-reared, obtained from a commercial supplier, etc.), and the approximate age of queens used in each study, 5) the temperature and relative humidity that the queens were exposed to during diapause, and 6) any other details related to the experimental design (for example, if queens were fed pesticides or exposed to infectious agents prior to treatment, etc.). For papers that did not report monthly survival estimates directly in the text of the manuscript, we used the digitize package in software program R (version 4.0.2) to extract data from figures. For three studies written in Korean, we extracted data from figures and English abstracts (Yoon et al. 2004, 2008, 2014). For these, we used the median number of queens per experiment (across all studies) as the sample size, since sample size was included in the abstract or figures. For two studies that monitored diapausing queens continually and reported the length of time queens survived rather than monthly survival rates (Lindsay 2020, Treanore and Amsalem 2020), we estimated monthly survival rates manually by extracting data from figures.

Many of the studies included in this literature review performed treatments on queens which were likely harmful (e.g., inoculating queens with parasites, exposing queens to chemicals, monitoring queens that were >30 days old at the start of diapause). We excluded all data from treatments we deemed obviously harmful, including only measurements for bees that were overwintered in continuous darkness, at constant temperatures between 1-5°C (see Appendix 5.1: Table S5.1 for further detail). Queens from excluded treatments had lower survival than those included in our meta-analysis (see Appendix 5.1: Figure S5.1).

We used a generalized linear mixed effects model (binomial family, logit-link), with queen survival coded as the response to estimate diapause mortality rates (prior to statistical analysis, we converted survival rates recorded from manuscripts to a binomial data set from sample sizes and percent survival at each time interval). GLMMs were fit using the command glmer() in R package lme4 (Bates et al. 2015).

We first fit a GLMM that included diapause interval as a fixed effect and random slopes and intercepts for both study species and paper ID. This model failed to converge, as there was not enough data to estimate variances for all random effects in the model (specifically, the correlation between the random slope and random intercept of species was equal to 1). Given that 100% of the queens were alive at the start of each study (i.e., Diapause period = 0), we might expect the random intercept of species to be less variable than the slope. Thus, we excluded the random intercept of species from the final model. As a basic check of model fit, we used a linear model to compare the observed and the predicted values, and estimated confidence intervals for the slope and the intercept of this model (if the original model is a good fit for the data, the linear model fit to the predicted values should have a slope of ~ 1 and intercept of ~ 0).

Results

Field study

Over the course of the study period, we excavated 141 potential overwintering sites, unearthing 111 diapausing queens (Table 5.1). Queens buried themselves anywhere from 2.5 to 11 cm below the ground, and larger queens buried themselves more deeply in the soil (See Table 5.1, Appendix 5.2).

At 2 months, 93.7 ± 2.3% of recovered queens were alive (Figure 5.2). Estimates of survival at 2 months did not differ between aggregations (binomial GLM, χ^2= 5.161, df = 3, P = 0.160), ranging between 88.0±8.0 and 100.0±0.0%.

At 6 months, 72.1±4.8% of queens recovered during the study were alive (though we failed to recover an additional 8.2% of queens in the spring). Thus, even by our most conservative measure, 63±4.8% of queens survived the 6-month diapause period (Figure 5.2). Estimates of mortality at 6 months were dependent on aggregation ID: the effect of aggregation ID was significant for both the model fit to data with unrecovered queens excluded (binomial GLM, χ^2= 11.318, df = 3, P = 0.010), and the model fit to data with unrecovered queens included (binomial GLM, χ^2= 12.739, df = 3, P = 0.005). Queens from aggregation 1 had the highest mortality rates (59.1±10.0%), followed by aggregation 4 (50.0±10.1%), 2 (23.3±7.7%), and 3 (17.4±7.9%) (unrecovered queens included in estimates of mortality).

When data were pooled across aggregations, IT span was a marginally significant predictor of queen fate (multinomial logistic regression, χ^2= 5.64, df =2, P = 0.059): larger queens were more likely to be recovered as live individuals, whereas smaller queens were more likely to be recovered dead and to not be recovered at all. Aggregation ID was also a marginally significant predictor of queen fate (multinomial logistic regression, χ^2= 5.64, df =2, P = 0.052), when the effect of IT span was not included in the model. Queens from aggregations 2 and 3 were the most likely to be recovered live (81.9±8.2% -84.6±7.1%), followed by queens from aggregation 4 (57.9±11.3%) and aggregation 1 (48.0±11.1%). However, when IT span and aggregation ID were assessed as additive effects, neither predictor was significant ([IT span, multinomial logistic regression, χ^2= 0.481, df = 2, P = 0.786], [aggregation ID, multinomial logistic regression, χ^2= 7.285, df = 6, P = 0.295]). This result implies that our data do not have the power to distinguish between effects of IT span and other factors that differ among aggregations.

Meta-analysis

Diapause survival rates for 5 species of bumble bee (*B. huntii*, *B. ignitus*, *B. impatiens*, *B. terrestris*, *B. vosnesenskii*) were extracted from the literature. *B. terrestris* was the most commonly studied species: 13 of 15 studies evaluated used this species (Appendix 5.1, Table S5.1).

The model used to predict survival as a function of diapause period seemed to be a good fit for the data: the intercept of the linear model fit to predicted values approximated 0 (-0.012, 95% CI: -0.061-0.037), and slope of the line approximated 1 (1.02, 95% CI: 0.94-1.10). On average, queen survival after 2 months was 74.8%, but dropped to 10.4% after 6 months (Figure 5.2). Predictions varied considerably across studies (Figure 5.2): of the random effects included in the model, the random effects associated with paper ID had the largest standard deviations

(0.834 for the random intercept and 0.438 for the random slope). In comparison, the estimated standard deviation for the random effect of species (slope) was 0.213 (note: standard deviations are calculated on a logit-scale).

Discussion

Understanding how mortality rates estimated in the lab compare to estimates of survival in natural populations is an important step in identifying sensitive life cycle stages. Laboratory studies of *Bombus* diapause have indicated that many queens are unable to survive the natural length of diapause (~ 10% survival after 6 months, Figure 5.2). Here, we observed that > 60% of *B. impatiens* queens monitored in the field survived a 6-month period of diapause (Figure 5.2). This estimate is higher than predicted for any laboratory study of *B. impatiens*, as well as most laboratory studies of other *Bombus* species (Fig 2). Our field estimates of survival are comparable to those of Pouvreau (1970), who reported survival rates of 68-72% for *B. agrorum*, *B. hypnorum*, *B. lapidarius*, *B. pratorum*, and *B. terrestris* queens overwintered in semi-natural conditions (for the natural length of diapause). Together, these studies suggest that surviving diapause may be less of an ecological hurdle for queen bumble bees than previously indicated.

Disparities in diapause survival rates between lab and field studies could arise for several reasons. Laboratory conditions can be stressful, and the ability of study subjects to thrive in the lab can depend greatly on methods of husbandry implemented by researchers (Yearian and Wilkinson 1965). The study subjects used in lab and field experiments could also differ in and of themselves – although the use of lab-reared insect populations is extremely common in ecological and physiological research, lab populations may adapt to laboratory conditions rapidly (Hoffmann and Ross 2018).

Bombus queens used in diapause experiments are often obtained from colonies purchased from commercial bumble bee suppliers (Koppert Biological Systems, Biobest, etc.). To date, no study has addressed the impacts of commercial rearing on the ability of queens to complete diapause directly. However, among other insect taxa (parasitoid wasps, lygus bugs), laboratory rearing has been observed to disrupt the ability to enter diapause, likely because of relaxed selection (Brent and Spurgeon 2011, Gariepy et al. 2014). For bumble bees, lab rearing can lead to other morphological changes that might impact overwinter survival. Beekman et al. (1998), for example, noted a decrease in queen body size over a multi-year experiment on queen diapause – after 4 years of captive rearing, no queens with a wet weight > 1 gram were produced (Beekman et al. 2000). Body size (e.g., weight) is frequently cited as an important driver of diapause survival for queen bumble bees (Bogo et al. 2017, Fauser et al. 2017). Queens under a certain size threshold (0.4 g for *B. lucorum*, 0.6 g for *B. terrestris*) seem to be unable to survive through diapause (Beekman et al. 1998, Vesterlund et al. 2014), leaving authors to ponder why colonies would produce such small queens (c.f. Beekman et al. 1998). However, it may be that small queens are an artifact of commercial rearing. Given that commercial rearing of bumble bees is known to impact both colony development (Gosterit and Baskar 2016) and worker morphology and behavior (Pugesek et al. *in press*), determining if rearing practices impact the ability of queens to diapause would certainly be a valuable area of future research.

Our results broadly corroborate an important pattern observed by the majority of lab-based studies of queen diapause survival (Bogo et al. 2017; Fauser et al. 2017), in that we observed a positive trend between queen body size and the probability of surviving until March (Figure 5.3). Although it is the first of its kind, our study included only four aggregations (with greater variation in body size among than within aggregations). Therefore, it was not possible to determine whether these differences are inherent to larger queens, or if this trend arose because of colony-level differences in body condition or differences in environmental-conditions around nest sites (e.g., soil texture). Behavioral differences between large and small queens could have also impacted survival to some extent, as larger queens also tended to bury themselves more deeply in the soil (see Appendix 5.2). In this context, our research does point indirectly to at least one major advantage of monitoring queen vital rates in the lab. When it is possible to control extraneous sources of variation (e.g. Nasir et al. 2019), identifying the environmental factors which impact queen vital rates is more straightforward.

Together, our findings emphasize the importance of utilizing both field- and lab-based methods in the study of insect overwintering ecology. Diapause is an incredibly understudied phase of the life cycle for bumble bees, as well as most other insects. Lab-based studies will remain foundational in the effort to identify the environmental factors which impact diapause survival: there are certainly obvious advantages to monitoring bumble bee vital rates in controlled conditions. However, as emphasized by our study, pairing controlled laboratory studies with field-based research can give a more complete picture of the real-world environmental pressures faced by diapausing insects.

Acknowledgements

This project was performed with the support of the Trustees of Reservations. We thank Nick Dorian and Sylvana Finn for their assistance collecting field data.

Tables

Table 5.1. General descriptions of the locations of overwintering queens around nest entrances.

Aggregation ID	Year	Number of sites found	Number of sites excavated by researchers	Proportion of sites that were abandoned by queens	Mean distance of sites from nest entrances (m)	Range of depths at which queens were buried (cm)
1	2019	92	36	0.389	2.03	3.0-7.5
2	2019	103	38	0.131	1.56	4-11
3	2020	101	33	0.121	1.63	4.5-10.5
4	2019	39	34	0.205	1.54	2.5-8.2

Figures

Figure 5.1 A) Soil displaced by an overwintering queen excavating her hibernaculum, B) a queen bumble bee excavating her hibernaculum, C) the remains of a dead queen, displaced from her hibernaculum, and D) a live queen, displaced from her hibernaculum.

Figure 5.2 Queen diapause survival as a function of time. Predictions generated from lab-based studies of queen diapause survival are represented by lines: the dark green line represents average estimates of diapause survival and each black/gray line represents the predicted estimate of diapause survival for a single study (black lines represent studies that used *B. impatiens*, whereas gray lines represent studies that used a different *Bombus* spp). Estimates of survival from our field study are represented by points: dark blue points represent estimates of diapause survival generated from pooled data (error bars represent standard errors), whereas light blue points represent estimates of survival for each aggregation. The shape of each point is indicative of how survival was calculated (circles = queens that were not recovered during field work were included in estimates of mortality: triangles = queens that were not recovered were excluded from estimates).

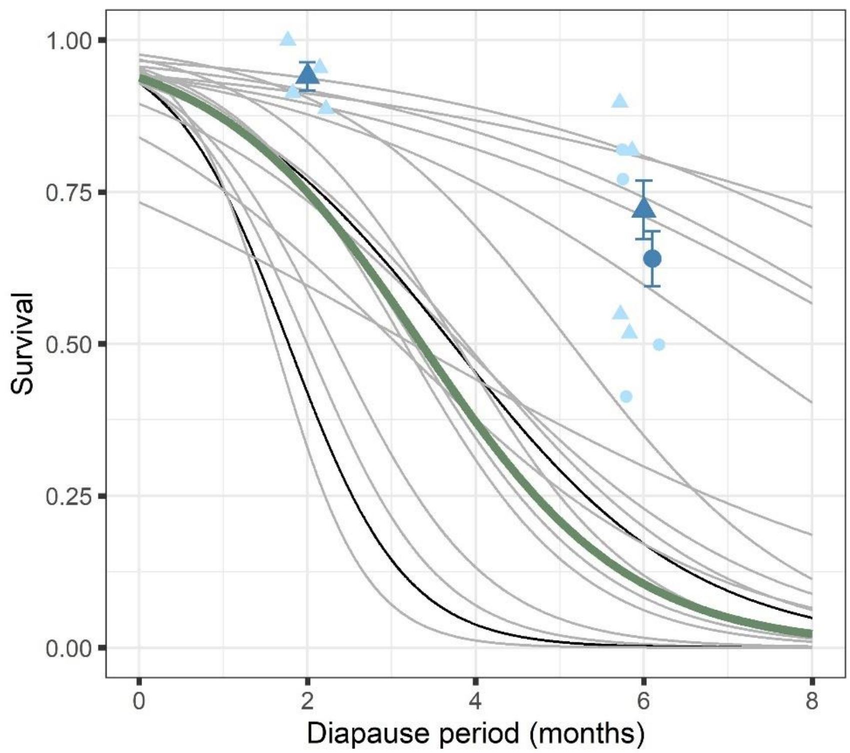
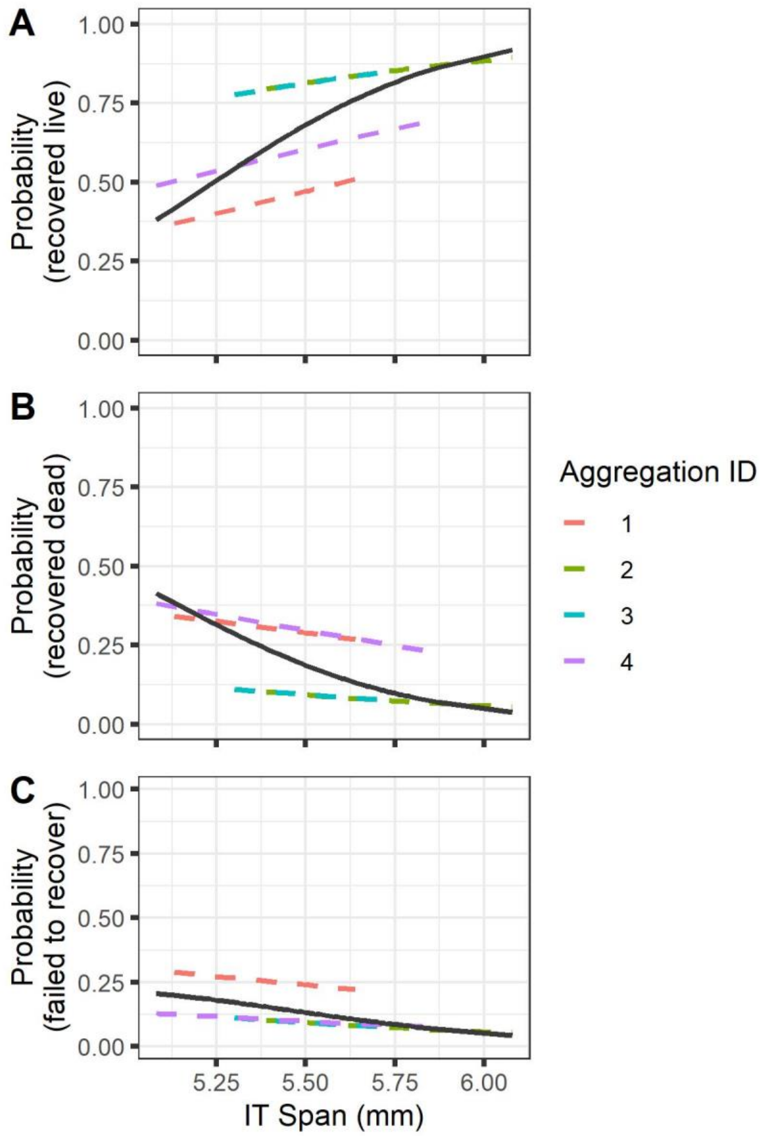


Figure 5.3. Spring fate of queens as a function of intertegular (IT) span. Black, solid lines represent probabilities of A) recovering a live queen B) recovering a dead queen or C) recovering no queen at all, as estimated from data pooled across aggregations. Dotted, colored lines represent estimates of probability calculated by accounting for aggregation ID as an additive effect.



Appendix 5.1

The methodologies of studies obtained via Web of Science were remarkably similar, in that queens overwintered in the lab were generally stored in incubators from anywhere from 1-6 months, at temperatures between 0°C and 5°C, and were kept at a RH of 50%-70%. However, as the purpose of many of these studies was to test the impacts of environmental stressors on queen physiology and survival, many queens were also kept at less-than-ideal conditions. We did not want to include estimates of diapause survival that would not be representative of conditions typically experienced by bees in the lab. Thus, only queens that were overwintered in continuous darkness, at constant temperatures between 0 and 5°C (hereafter called the "control" group) were included in our final data set, whereas queens which were manipulated in a way that was deemed obviously harmful, or kept at temperatures <0°C or >5°C (hereafter called the "treatment" group) were excluded (see Table S5.1) (a detailed description of the data excluded for each study is provided in Table S5.1).

To determine if survival rates differed between queens in "control" and "treatment" groups, we used a generalized linear mixed effects model (binomial family, logit-link). This GLMM was similar to the GLMM used to predict bumble bee diapause survival rates (i.e. the model used in the meta-analysis). We used the same random effect structure (the model included random slopes for species and paper ID, and a random intercept for paper ID). We also included both diapause period and treatment as fixed effects, as well as an interaction term. The significance of each fixed term was determined using Type II marginal hypothesis tests implemented with the Anova() function in the car package (Fox and Weisberg 2019).

Queens in the "control" group generally had higher rates of survival than queens in the "treatment" group (Figure S5.1). We identified both diapause period and treatment as significant predictors ([diapause period, GLMM, χ^2= 26.139, df = 1, P < 0.0001], [Treatment, GLMM, χ^2= 180.001, df = 1, P < 0.0001]). There was, however, no significant interaction between the two fixed effects (GLMM, χ^2= 0.448, df = 1, P = 0.503).

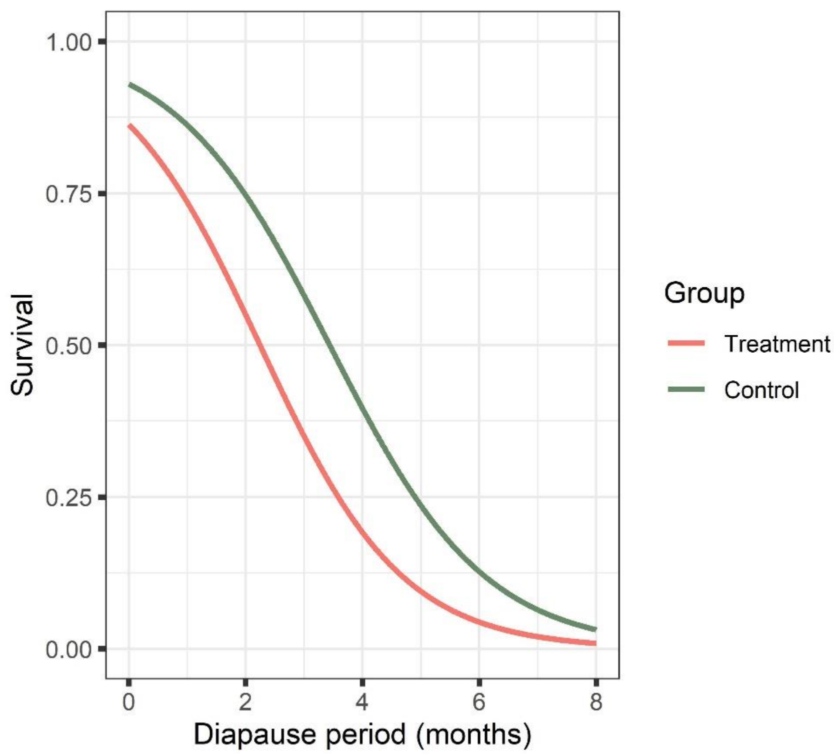
Table S5.1. For each study reviewed, a brief description of data (if any) excluded from meta-analysis.

Study	Queen origin	Species	Data excluded
Beekman et al. 1998	Lab-reared	*B. terrestris*	Queens overwintered at temperatures below 0°C and 5°C
Bogo 2016*	Commercial	*B. terrestris*	NA
Bogo et al 2017	Commercial	*B. terrestris*	None
Bortolotti et al. 2020	Commercial	*B. terrestris*	None
Fauser et al. 2017	Lab-reared	*B. terrestris*	Queens exposed to neonicotinoids or parasites
Gosterit and Gurel 2009	Lab-reared	*B. terrestris*	None
Lindsay 2020	Both lab-reared and commercial	*B. huntii*, *B. impatiens*, *B. vosnesenskii*	Queens exposed to temperature flux treatment
Nasir et al. 2019	Commercial	*B. terrestris*	Queens overwintered at temperatures below 0°C
Ozsahin and Gosterit 2018	Lab-reared	*B. terrestris*	None
Treanore & Amsalem 2020	Commercial	*B. impatiens*	None
Vesterlund et al. 2014	Lab-reared	*B. lucorum*	None
Woodard et al. 2019	Commercial	*B. terrestris*	Queens which were nectar or pollen starved, or were fed low- or high-sucrose nectar prior to diapause
Yoon et al. 2004*	NA	*B. terrestis*, *B. ignitus*	NA
Yoon et al. 2008	Commercial	*B. terrestris*	None

Yoon et al. 2010a	Lab-reared	*B. terrestris*	Queens overwintered at temperatures above 5°C
Yoon et al. 2010b	Lab-reared	*B. terrestris*	None
Yoon et al. 2013	Lab-reared	*B. ignitus*	Queens overwintered at temperatures above 5°C
Yoon & Lee 2014	Lab-reared	*B. ignitus*	Queens aged > 30 days (before being placed in incubators)
Yoon et al. 2014	NA	*B. ignitus*	None

*The data relevant to our meta-analysis was also presented in another paper.

Figure S5.1. Diapause survival as a function of time for queen *Bombus* overwintered under "control" conditions (i.e., queens were overwintered in continuous darkness, at constant temperatures between 0 and 5°C) and queens overwintered under "treatment" conditions (i.e., queens that were manipulated in a way that was deemed obviously harmful, or kept at temperatures <0°C or >5°C).



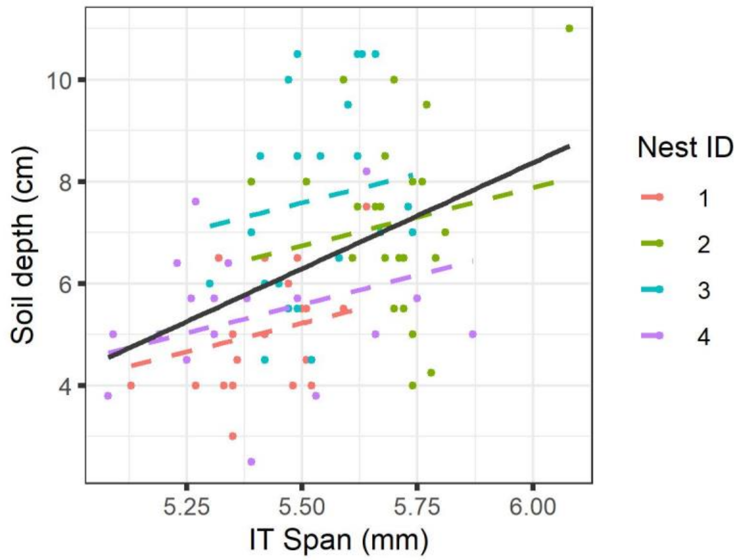
Appendix 5.2

For other insect taxa, body size may influence pre-diapause behavior. Large Colorado potato beetles (*Leptinotarsa decemlineata*), for example, tend to bury themselves in the soil, and have higher rates of survival compared with smaller beetles that remain on the ground surface (Piiroinen et al. 2011). Similarly, larger spruce web-spinning sawfly (*Cephalcia arvensis*) larvae tend to bury themselves more deeply in the soil (Batisti 1994). Here, we explore if similar patterns arise for bumble bees.

Due to low sample sizes, we ran three separate linear models to explore the relationships between hibernaculum depth, IT span, and aggregation ID. First, we pooled data across aggregations, and ran a model that included IT span as a predictor and hibernaculum depth as the response. Second, we ran a model that included aggregation ID as a predictor, and hibernaculum depth as the response. Third, we ran a model that included additive effects of both aggregation ID and IT span as predictors. Linear models were fit using the command lm(), a base function in R. The significance of each fixed term was determined using Type II marginal hypothesis tests implemented with the Anova() function in the car package (Fox and Weisberg 2019).

When data were pooled across aggregations, IT span was a significant predictor of hibernaculum depth (LM, $F = 17.37$, df =1, $p < 0.0001$). Larger queens were more likely to overwinter more deeply in the soil (Figure S5.2). Aggregation ID was also a significant predictor of depth (LM, $F = 13.95$, df =1, $p < 0.0001$): queens from aggregation 3 overwintering deepest in the soil (7.6±0.3 cm), followed by queens from aggregation 2 live (7.2±0.3 cm), aggregation 4 (5.4±0.4) and aggregation 1 (5.1±0.4 cm). When IT span and aggregation ID were assessed as additive effects, the effect of aggregation ID was significant (LM, $F = 8.42$, df =1, $p < 0.0001$), and the effect of IT span was marginally significant (LM, $F = 3.87$, df =1, $p = 0.053$). It is worth noting that both aggregation 1 and aggregation 4 were located < 1m from walking paths: compacted soils may have limited the ability of queens to dig themselves very deeply (a greater number of hibernacula were abandoned by queens at these aggregations; see Table 5.1 of the main text).

Figure S5.2. The depth at which queens were buried, as a function of intertegular (IT) span. Black, solid lines represent the relationship between soil depth and IT span, estimated by pooling data across aggregations, whereas dotted, colored lines represent estimates, were calculated by accounting for aggregation ID as an additive effect.



Chapter 6:
Supplement: Accounting for imperfect detection in species with sessile life cycle stages: a case study of bumble bee nests.

Published in the *Journal of Insect Conservation*:

David T. Iles, Genevieve Pugesek, Natalie Z. Kerr, Nicholas N. Dorian, and Elizabeth E. Crone (2020) Accounting for imperfect detection in species with sessile life cycle stages: a case study of bumble bee nests.

Abstract

1. For bumble bees and other social organisms, colonies are the functional unit of the population rather than the individual workers. Estimates of bumble bee nest density are thus critical for understanding population distribution and trends of this important pollinator group. Yet, surveys of bumble bee nests and other taxa with sessile life stages rarely account for imperfect detection.
2. Here, we demonstrate the use of mark-recapture methods to estimate the density of bumble bee nests at multiple sites using standardized survey protocols. We detected ~30% of nests in a two-hour survey of each 3000 m^2 plot.
3. We determined that 4-5 visits were sufficient to estimate the total number of nests at our site with reasonable precision, equating to one-third the effort previously assumed necessary to reliably estimate nest density. Mark-recapture approaches can be used to generate unbiased estimates of density with reduced search effort, while simultaneously increasing the rate at which nests are discovered.

Introduction

Measuring the size of natural populations is a primary goal of conservation monitoring and is often a prerequisite for management action or wildlife policy decisions (Williams et al. 2002). Yet, estimating abundance and the factors that influence it can be challenging when organisms are difficult to detect. Accordingly, study designs and analyses that account for imperfect detection have a rich tradition in wildlife research (White and Burnham 1999, Williams et al. 2002, Kéry and Schaub 2012). Failure to account for imperfect detection of cryptic organisms can introduce both uncertainty and bias into population estimates, potentially leading to erroneous conclusions about population status and habitat requirements (Gu and Swihart 2004, Kéry and Schmidt 2008).

In contrast to studies of mobile vertebrates, imperfect detection is rarely accounted for in population estimates of both invertebrates and taxa that are sessile or have a sessile lifecycle stage, including plants and ground-nesting animals (Kellner and Swihart 2014, Berberich et al. 2016). Kellner and Swihart (2014) found that only 9.0% of invertebrate and 1.4% of plant population studies accounted for imperfect detection, compared to the mean of 23% across all taxa. This discrepancy potentially stems from the incorrect assumption that detection probability is inherently high in less mobile organisms. For example, targeted surveys of milkweed plants (Slade et al. 2003), caterpillar aggregations (Brown et al. 2017), and ant nests (Berberich et al. 2016) have reported detection probabilities well below 0.50. Thus, detection can be highly imperfect and systematically biased for sessile organisms that are rare, inconspicuous, or logistically difficult to survey.

At the present time, monitoring studies of bumble bees are typically based on lethal collection of foraging workers at floral resources. This approach is problematic because bumble bees are social insects and the demographic unit of interest is often the colony (Goulson 2010). Studies of nesting habitat are therefore particularly valuable for conservation planning. Yet, bumble bee nests are difficult to find, and relatively few studies have investigated the correlates of nest density of wild bumblebees compared to the numerous studies using foraging workers as a metric of habitat quality (but see examples in Harder 1986, Osborne et al. 2008b, Waters et al. 2010, Lye et al. 2012, O'Connor et al. 2012, O'Connor et al. 2017). This disparity is problematic because workers are highly mobile and can forage up to 11 kilometers from their nest (Greenleaf et al. 2007; Rao and Strange 2012; Pope and Jha 2018) and may be attracted to areas rich in floral resources. This decoupling of foraging and nesting sites potentially obscures the landscape drivers of population performance (Heard et al. 2007). Indeed, Herrmann et al. (2007) reported that bumble bee nest densities were uncorrelated with local worker densities across an agricultural landscape in Germany.

A variety of methods have been used to estimate the density of bumble bee nests across landscapes. These methods have included "free searches" in which observers haphazardly search particular habitat types for nests (Harder 1986, O'Connor et al. 2012, Rao and Skyrm 2013, O'Connor et al. 2017), canine-assisted searches (Waters et al. 2010, O'Connor et al. 2012), and "fixed searches" in which concentrated stationary observation of small plots are performed by individual researchers or distributed citizen science networks (Osborne et al. 2008b, Lye et al. 2012). As an alternative to ground-based searches, molecular (i.e., genetic) analysis of foraging workers has also been used to infer nest density at large spatial scales (Darvill et al. 2004, Goulson et al. 2010, Rao and Strange 2012). Colonization of artificial next boxes has been used to assess some aspects of habitat selection (Hobbs 1964, Hobbs 1965, Richards 1978), but have not been widely applied to studies of nesting bumble bee abundance, due in part to very low colonization rates and possible biases associated with nesting in boxes (cf. Richards 1978, Fussell and Corbet 1992). Other studies have inferred the relative density of nests across space based on the prevalence of spring nest-searching behavior by newly emerged queens (Svensson et al. 2000, Kells and Goulson 2003, O'Connor et al. 2017). Combined, these studies have reported considerable variation in nest densities (range: 0.1 to 50.1 nests$\cdot$ ha^{-1}; Table S6.1 in Appendix 6.1), potentially due to ecologically relevant differences across species, habitat types, and landscape configurations. However, O'Connor et al. (2012) also reported a 20-fold difference in the number of nests detected between fixed and free searches at the same site, indicating an extreme degree of variation in nest detection among survey strategies. Unaccounted differences in detection probability within and among studies could contribute strongly to observed variation in nest density, limiting the ability to generalize across studies and resolve the true environmental correlates of bumble bee population abundance.

Here, our objective is to demonstrate the use of mark-recapture techniques for estimating the density of bumble bee nests in a field setting. We specifically target a period of the annual cycle during which colony establishment and failure is rare, allowing us to apply closed population models to measure and remove systematic bias in nest detection. This approach provides a formal framework for generating unbiased estimates of nest density, thereby facilitating valid comparisons of density across sites and studies. We also illustrate how mark-recapture approaches can be used to optimize survey design by reducing the effort necessary to reliably estimate density, while simultaneously increasing the rate at which nests are discovered. This approach can easily be generalized to species with different nesting ecologies, and we conclude by discussing related approaches when the number of nests changes during the survey period.

Methods

Study Site

We conducted searches for bumble bee nests in three survey plots located at Appleton Farms (42.65°N, 70.86°W) in Ipswich, Massachusetts (Figure 6.1). Each plot was approximately 3000 m^2 in area and not used for agriculture, although each plot is mowed annually to prevent succession. Two plots were adjacent to one another, while the third plot was located approximately 1 km away. Primary vegetation cover in each plot consisted of a variety of grasses, sedges (*Carex* spp.), perennial forbs (e.g., *Plantago lanceolata*, *Linaria vulgaris, Lotus corniculatus*, *Asclepias syriaca*, *Solidago* spp.), and shrubs (e.g., *Vaccinium angustifolium, Rubus* spp.). Each plot was bordered by a hedgerow of trees or forest, and the surrounding landscape was mixed agriculture (pasture and hay fields) and natural areas (forest and wet meadows). *Bombus impatiens* is the most common species at this site, and for this reason, we focus our study on this species, though *B. bimaculatus* and *B. griseocollis* are also frequently encountered. All three species primarily nest in pre-existing cavities in the ground or on the ground surface (Plath 1922, Laverty and Harder 1988).

To demonstrate the application of mark-recapture techniques to surveys of bumble bee nests, we focused our surveys on areas with high probability of containing ground-nesting bumble bee colonies based on prior knowledge of site conditions. However, mark-recapture methods are also useful in cases where spatial patterns in species occurrence is unknown (discussed further in *Discussion*, below).

Data Collection

Each plot was visited repeatedly in July and August of 2017, after bumble bee colonies had produced several cohorts of workers. This time of year corresponds to the period of peak worker density for *B. impatiens*, and occurs after *B. griseocollis* nests have largely senesced (note that we did not discover any *B. griseocollis* nests; see *Results*). New nests are unlikely to be initiated during this period, and we did not observe any queens searching for nests across the range of survey dates. Nest failure is also extremely rare over this period; most mortality of *B. impatiens* occurs in early June during colony establishment and colonies typically do not senesce until early September (G. Pugesek unpublished data).

We conducted surveys opportunistically during clear weather conditions between 6:30 am and 7:30 pm, though the majority of nest surveys took place in the morning. Searches were conducted independently by eight different investigators, several of whom surveyed each plot multiple times (range: 1–3 times per person). Each plot was surveyed for two hours between 12–16 times, for a total effort of 24–32 survey hours per plot.

Our mark-recapture study entailed repeated surveys of plots to determine the probability of discovering new nests and relocating previously discovered nests; ultimately, this allows us to estimate the number of undiscovered nests in each plot. During each survey, searchers moved slowly through the plot looking for bumble bee activity that might indicate the presence of nest (i.e., workers quickly descending to the ground, slowly ascending from the ground, or conducting circular navigation flight behavior). We confirmed the presence of a nest by locating the entrance hole and monitoring worker traffic over a 5-20 minute period (Figure 6.1 c,d). Upon locating a bumble bee nest, searchers placed an inconspicuous, numbered identifier next to the nest entrance (for previously undiscovered nests) and recorded the nest location, species and nest identity, and whether the nest had been previously located either by themselves or by other searchers. For each located nest, we also recorded the height of the tallest vegetation immediately above the nest entrance, which could affect searcher ability to detect bumble bee movement near nest entrances. This covariate was measured approximately one month after completion of mark-recapture surveys, and we were unable to locate two nests at this point; for these nests, we imputed a mean height value for use in analyses. We also extracted other covariates that could plausibly influence nest detection, including hourly ambient air temperature measurements during each survey from the nearest weather station (approx. 9.5 km away); ambient air temperature could affect bumble bee metabolism and nest activity, which could influence our ability to detect nests. Finally, we also recorded the time of day of each survey, as bumble bee activity varies throughout the day (Kwon and Saeed 2003).

Description of Mark-Recapture Approach

We used closed population models to analyze the mark-recapture data because our surveys were conducted during a period in which nest initiation and mortality for our primary target species (*B. impatiens*) was unlikely. Closed population models assume the abundance of nests within each plot does not change across sampling periods (i.e., the population is demographically “closed” during the study); thus, variation in the number of nests detected across repeated surveys is caused entirely by observation error. The goal of closed population models is to estimate detection probability (p) of nests along with the spatial, temporal, and/or individual-level factors that influence it. Once the factors that influence detection probability are estimated, the observed count of individuals can be corrected to generate an unbiased estimate of the true number of nests present at a site.

The foundation of mark recapture approaches is the encounter histories of individuals (in this case bumble bee nests) that are generated from repeated surveys of plots. An encounter history of '0110' implies the nest was detected on the second and third survey of the plot and not detected on the first and fourth survey. Successful detection of the nest i during each survey t occurs with some probability $p_{i,t}$ and conversely, the probability of not detecting the nest is $1 - p_{i,t}$. The entire encounter history '0110' for nest i therefore occurs with probability $(1 - p_{i,1}) \times p_{i,2} \times p_{i,3} \times \left(1 - p_{i,4}\right)$. Model-fitting approaches (i.e., maximum likelihood estimation or Bayesian methods) are then used to estimate the values of $p_{i,t}$ that are most consistent with the data. Additionally, a link function can be used to model the influence of covariates on detection probability, analogous to a logistic regression.

Otis et al. (1978) outlined a classic catalogue of model structures that can be used to examine drivers of variation in detection probability for closed populations. We adopt this framework for our analysis of bumble bee nests to demonstrate how these models can be applied to studies of sessile organisms and to link our specific study system to a well-defined body of mark-recapture research. Mathematical descriptions of each model are presented in Table 6.1.

The simplest model, M_0, estimates a single detection probability that is common across all nests and sampling occasions. However, in reality, nests may differ in their probability of being detected (e.g., due to unmeasured differences in worker activity level or surrounding vegetative cover), a phenomenon known as individual heterogeneity. Consequently, the model M_H estimates both a mean and variance in detection probabilities across nests (i.e., individual random effects). Similarly, the model M_T estimates a mean detection probability across nests and a variance in detection associated with visits (i.e., a temporal random effect). This model structure allows for variation in detection probability among visits without requiring us to specify any systematic temporal pattern in detection probability. For bumble bees, this could be due to temporal variation in colony size, unmodeled effects of weather, or seasonal changes in vegetation within plots that alter the probability nests will be detected on each visit.

Fixed effects of explicit covariates can also be incorporated to examine the drivers of variation in detection probability (Kéry and Schaub 2012). These can include age or size of the individual (or nest), habitat covariates, or explicit temporal covariates (e.g., to examine temporal trends in detection). In our study, we constructed five additional models that included explicit covariates we hypothesized could affect the detection of bumble bee nests. The first of these models, M_b, accounts for a discrete behavioral change in organisms that affects their individual detection in subsequent marking occasions (Otis et al. 1978), commonly referred to as "trap-shyness" or "trap-happiness". In studies of sessile organisms, rather than behavioral changes of the study organisms themselves, this response can plausibly occur if the vegetation surrounding focal organisms becomes trampled by researchers or if researchers remember the location of individuals (in this case, nests) they have previously located. Either of these scenarios would result in different detection probabilities for initial and subsequent capture events in studies of sessile organisms. Additional models included a discrete effect of survey plot (M_{plot}), and continuous effects of vegetation height above the nest (M_{veg}), hour of the day at which plots were surveyed (M_{hour}), and ambient air temperature during the survey (M_{temp}). Our objective was to demonstrate how each of these covariate effects could be incorporated to generate deeper insights into the processes influencing nest abundance surveys, rather than to exhaustively examine the diverse suite of (potentially interacting) factors that influence nest detection.

Model Fitting

We fit this series of closed population models using the empirical nest encounter histories generated by our repeated surveys of plots. We fit models using Bayesian methods, outlined by Kéry and Schaub (2012, ch 6); note that such models can also be fit in a frequentist framework using maximum likelihood approaches. Bayesian analysis allows for random effects to be easily incorporated and for uncertainty in parameter estimates to be easily propagated to the model output. The Bayesian implementation of closed population models uses an additional technique called "data augmentation" to estimate the true number of individuals in a plot based on the number of nests actually detected and their estimated detection probabilities (see Kéry and Schaub 2012, ch. 6; Royle and Dorazio 2008, section 5.6 for further discussion of this technique). Data management and simulations were conducted in R version 3.4.4 and Bayesian analysis was conducted in JAGS using the *jagsUI* library in R (Kellner 2018).

Results

We located 18 bumble bee nests across the three survey plots (10, 5, and 3 nests in each plot, respectively). All nests were constructed by *Bombus impatiens*, except for one that was constructed by *B. bimaculatus*. We used all nests for subsequent analysis. The number of nests located on single visits to each of the three plots ranged from 0 to 6, 0 to 3, and 0 to 3 for each plot, respectively. The three plots were searched 11, 16, and 14 times by at least 6 different observers.

Model results – patterns in nest detection

The mean detection probability, p, of nests based on the intercept-only model (M_0) was 0.30 (95% credible interval [CRI] = 0.24–0.36; Figure 6.2a). A model that included heterogeneity in p across nests (M_H) suggested that nests differed in their individual detection probabilities, with a median posterior estimate for p of 0.26 (95% CRI = 0.11–0.37) and standard deviation (on the logit scale) of 0.79 (95% CRI = 0.14–1.84; Figure 6.2b). Similarly, a model that included heterogeneity in p across visits (M_T) indicated that detection probability varied through time with a median p of 0.27 (95% CRI = 0.20–0.38) and standard deviation (on the logit scale) of 0.67 (95% CRI = 0.10–1.29; Figure 6.2b). We note that there was substantial uncertainty associated with estimates of both individual and temporal random effects, as is common for random effect models fit to relatively sparse data (Kéry and Schaub 2012).

We evaluated the effects of five additional covariates on p. There was weak evidence for different detection probabilities between the first and subsequent capture occasions (model M_b; Figure 6.2c). Thus, nests were not more likely to be detected after their initial discovery. A model including different detection probabilities for nests within each survey plot (M_{plot}) indicated nest detectability varied systematically across plots (Figure 6.2d). Under this model, median estimates of detection probabilities in each plot were 0.36 (95% CRI = 0.27–0.45), 0.23 (95% CRI = 0.14–0.33), and 0.26 (95% CRI = 0.15–0.41). The probability that detection probability was greater for nests in plot 1 than plot 2 was 0.98 (calculated directly from posterior probability distributions). Height of vegetation above the nest entrance did not have a strong effect on p (standardized effect of vegetation height from M_{veg} = 0.02; 95% CRI = -0.02–0.06; Figure 6.2e). However, p declined strongly throughout the day during sampling times (standardized effect of hour from M_{hour} = -0.68; 95% CRI = -1.25– -0.25; Figure 6.2e) and was negatively correlated with ambient air temperature during the survey (standardized effect of air temperature from M_{temp} = -0.38; 95% CRI = -0.69– -0.09; Figure 6.2e).

Model results – nest abundance

Across all eight models, median estimates of nest abundance were in close agreement. All models estimated approximately 10, 5, and 3 nests in each of the three plots, respectively (Figure 6.3). The corresponding median estimate of nest density in each 0.3 ha plot was therefore 33.3, 16.7, and 10 nests·ha^{-1}, and average density across the three plots was 20 nests·ha^{-1}. Consequently, on single surveys of each plot, we located approximately 0–60% of the nests in plots 1 and 2, and 0–100% of the nests in plot 3.

However, even with our large number of repeated searches (11–16 per survey plot), there was a high probability that undiscovered nests remained in each plot at the end of our study (Figure 6.3; note range of credible intervals). For example, while the median estimate of total nest abundance from model M_0 was 18 (equal to the number of nests we located), the 95% credible interval was 18–20, and the probability that the true abundance was greater than 18 was 0.25. Notably, the credible intervals for estimates from model M_H were wide relative to other models. This reflects two important features of individual heterogeneity: 1) a fraction of nests have extremely low detection probabilities and it is difficult to estimate how many remained undetected, and 2) the existing amount of heterogeneity is difficult to estimate, especially with low sample sizes (see Figure 6.2b).

Lastly, to quantify the effort needed to reliably estimate differences in nest density between plots, we sequentially re-fit model M_0 for different numbers of visits. With our small number of plots and so few nests initially detected, the model would not converge with only 2 visits to each plot. This also occurred when models were fit with maximum likelihood in program MARK instead of using Bayesian methods (but see Appendix 6.2 where a simulation confirms that models converge with 2 visits to a larger number of sites). The model converged with 3 visits to each plot, but uncertainty associated with abundance estimates was extremely large (Figure 6.4). After 4 visits to each plot, clear differences in abundance between plots 1 and 3 were apparent. As expected, uncertainty in estimates continued to decline as the number of surveys increased.

Discussion

Our study is the first to apply mark-recapture methods to estimate the density of bumble bee nests, which represent a critical and understudied life cycle stage for this important pollinator group. In our study, single surveys of bumble bee nest abundance were subject to considerable bias and observation error, owing to imperfect detection. On average, we only detected 30% of existing nests on each 2-hour survey of a 0.3 ha plot. Raw counts from individual surveys were also highly variable, resulting from substantial sampling variation that obscures true differences in nest abundance among sites (Appendix 6.2). However, even after more than 11 surveys of a plot (equating to similar effort in common intensive "fixed search" survey approaches; O'Connor et al. 2008, Lye et al. 2012), there was a substantial probability that nests remained undiscovered. This indicates that even in labor-intensive surveys, raw counts can be biased by imperfect detection. In order to understand the nesting ecology and monitoring requirements of bumble bees, we must account for imperfect detection of nests.

In addition to generating unbiased estimates of nest density, mark-recapture provides a powerful approach for optimizing survey design to meet multiple objectives. Often, a secondary goal of monitoring is to locate and sample large numbers of individuals (e.g., to collect genetic data, assess physiological status, monitor behavior, investigate micro-habitat associations, etc.). In this case, labor-intensive fixed searches for bumble bee nests are inefficient (O'Connor et al.

2012) and logistically challenging to implement over large areas without distributed citizen science networks (Osborne et al. 2008b, Lye et al. 2012). For example, stationary fixed searches across a range of habitats in the UK resulted in average discovery rates of one nest every 170 minutes with a corresponding search effort of 0.47 min·m^{-2} (see Table 2 in Osborne et al. 2008b). Using mark-recapture, we generated precise and unbiased estimates of nest density with only four visits to each plot (Figure 6.3), corresponding to a search effort of 0.16 min·m^{-2}; approximately three times less than typical fixed search methods. Given four visits to each plot, average detection probability at our sites (p = 0.3), and average nest density (20 nests·ha^{-1}), we expect to discover one nest every 103 minutes. We therefore discovered nests at a comparable rate to low-intensity free searches (approximately one nest every 80 minutes; O'Connor et al. 2012), while simultaneously generating precise and unbiased estimates of nest density with substantially less effort than high-intensity fixed searches. In principle, unbiased estimates of density can be obtained with only two surveys per plot, though these estimates are imprecise (Appendix 6.2). As a preliminary recommendation, we encourage 3-4 visits/site (see Figure 6.4 and Appendix 6.2).

The range of bumble bee nest densities we detected are comparable to those reported in Osborne et al. (2008b), who used intensive fixed searches by citizen science volunteers to count nests in UK gardens and countryside habitats. Of our three plots, the highest density we detected was 33.3 nests·ha^{-1}, similar to hedgerow (29.5 nests·ha^{-1}), garden (35.9 nests·ha^{-1}), and fence line (37.2 nests·ha^{-1}) habitats reported in Osborne et al. (2008b). Notably, Cumber (1953) is the only other study to report higher nest densities than these; his estimate of 48.6 nests·ha^{-1} was based on intensive free searches of a refuse dump in England. Conversely, our lowest density plot contained 10 nests·ha^{-1}, which is comparable to the density of 10.9 nests·ha^{-1} reported in Harder

(1986) who intensively surveyed a successional field in Ontario, Canada. This estimate is also similar to the lowest densities in Osborne (10.8 nests·ha^{-1} in woodland and 11.4 nests·ha^{-1} in short grassland habitat). Therefore, our three plots seem to have captured the range of nest densities observed in other studies with intensive search effort, and thus high detection probabilities.

Other studies with lower search effort, and therefore differences in detection error, have reported far lower nest densities than those in our study or those in Osborne et al. (2008b), Cumber (1953), and Harder (1986). For example, low-intensity free searches by researchers or volunteers produced estimates of nest density ranging from 1.4–3.6 nests·ha^{-1}, similar to the range of nest densities discovered by bumble bee "sniffer dogs" (O'Connor et al. 2012, 2017). Both studies acknowledge that detection error is likely substantial for these methods. Molecular studies also typically yield estimates of nest density that are 1–2 orders of magnitude lower than intensive ground-based searches (range: 0.13–1.9 nests·ha^{-1}; Appendix 6.1). Several molecular studies have used ad-hoc approaches to account for imperfect detection, but these approaches likely underestimate the true nest density (Goulson et al. 2010). Molecular methods also integrate habitat quality over larger spatial extents than ground-based surveys, and likely incorporate areas that are unsuitable for nesting (e.g., water bodies), and also include a number of biases in terms of assigning nests to local habitat types (Pope and Jha 2017). Formal mark-recapture approaches are necessary to understand the degree to which variation in nest density between studies is driven by ecologically relevant factors (e.g., variation in habitat quality, differences in spatial scale at which studies occur) versus unresolved differences in imperfect detection.

We found that nest detection declined when surveys were conducted later in the day and in warmer temperatures (Figure 6.2e). Based on estimates from model M_{hour}, mean detection probability during 6 am surveys was 0.40 (95% CRI = 0.31–0.50) but was only 0.05 (95% CRI = 0.01–0.17) for surveys initiated at 6 pm. This result is consistent with Kwon and Saeed (2003) who found that colony traffic and foraging activity of *Bombus terrestris* declined throughout the day and when temperatures were warmer. Similarly, Couvillon et al. (2010a) reported that workers of all sizes conducted fewer foraging trips in warmer temperatures. Thus, the results of our mark-recapture estimate of detection probability are broadly consistent with prior knowledge of bumble bee foraging ecology and suggest that variation in forager behavior is a likely driver of differences in detectability.

Although we did not measure them in our study, other factors could also influence colony activity, and in turn, the probability that nests are detected on a given survey. For example, larger colonies have higher traffic at nest entrances (Kwon and Saeed 2003) and are therefore likely to be more detectable. Colony size, in turn, depends on floral resources available throughout the season (Williams et al. 2012, Crone and Williams 2016). Accordingly, detection of nests may differ between high- and low-quality habitat owing to systematic differences in colony size. Here, we found evidence for systematic differences in nest detection across our three survey plots. Plot 1 had the highest nest density (33.3 $nests \cdot ha^{-1}$), and simultaneously, detection was highest for nests in this plot (Figure 6.2d). Mark-recapture approaches can correct for these spatio-temporal biases in detection probability, which in turn, improve comparisons of abundance within and between studies.

We targeted a period of the season in which abundance of nests was unlikely to change between surveys, allowing us to take advantage of the conceptually simple (yet powerful) machinery of closed population models. However, alternative approaches are available when population closure is unlikely or if the goal is to explicitly measure changes in abundance within a season. Open-population models (Jolly 1965, Seber 1965, Schwarz and Arnason 1996) can be used to estimate abundance, survival, and recruitment simultaneously across intervals where individuals can enter or exit the population. Open-population models are especially useful when fit to data collected under a "robust" sampling design (Pollock 1982). In a robust design, multiple surveys are conducted over short time intervals during which population closure is assumed. This process is then repeated multiple times over longer intervals during which birth, immigration, death, and emigration can lead to changes in abundance. When analyzed together, these repeated surveys yield inference about the drivers of population change. Our bumble bee nest survey methods could accommodate this design by having independent observers survey the same plots within hours or days of each other, and then repeating this process multiple times throughout a season (e.g., every 1–2 weeks). The use of open population models to generate bias-corrected estimates of nest abundance, along with seasonal patterns of nest establishment, mortality, and senescence is therefore a ripe area of future research.

For most bumble bees, nesting ecology is poorly understood, which can hamper or even misguide conservation efforts of declining species. Our study offers one way to optimize surveys for wild bumble bee nests and generate precise estimates of nesting densities. Wild bumble bee communities also vary greatly across space and time (Williams 2001), patterns which could be due in part to unmeasured observation error and sampling variation (Link and Nichols 1994). Multi-species extensions to our approach could require adjustments to accommodate differences in nesting ecology, habitat preference, and phenology among species. Much of this information is unknown for declining species. Our surveying and modeling framework enables this information to be rapidly and precisely documented. Mark-recapture approaches could make studying bumble bee nesting ecology more feasible and lead to targeted population monitoring of this important group of species, as well as other insects with sessile life stages.

Acknowledgements

We thank the Trustees of Reservations and Appleton Farms for providing access to their properties where this study was conducted. We thank Russ Hopping for invaluable assistance in locating study sites. We also thank Annika Greenleaf, Max McCarthy, Moshe Steyn, Erin Wampole, and our sniffer-dogs-in-training, Indy and Molly, for assistance with field work. This work was supported by the US National Science Foundation (DEB1354022) and the US Strategic Environmental Research and Development Program (SERDP, RC-2119).

Tables

Table 6.1 Classic closed population model structures used in our study to examine variation in the probability of detecting bumble bee nests. $p_{i,t}$ refers to the detection probability of nest *i* on survey *t*, and β_i refer to various fitted model coefficients.

#	Model Abbrev.	Model Name	Formula	Description
1	M_0	Intercept-only	$logit(p_{i,t}) = \beta_0$	Constant value of *p* across all *i* nests and *t* visits
			Models with Random Effects	
2	M_H	Individual heterogeneity	$logit(p_{i,t}) = \beta_0 + \alpha_i$ with $\alpha_i \sim Normal(0, \sigma^2_{nest})$	Random effect for *p* among *i* nests
3	M_T	Temporal heterogeneity	$logit(p_{i,t}) = \beta_0 + \alpha_t$ with $\alpha_t \sim Normal(0, \sigma^2_{time})$	Random effect for *p* among *t* visits
			Models with Categorical Covariates	
5	M_b	Capture/recapture effect	$logit(p_{i,t}) = \beta_0 + \beta_1 * recap_{i,t}$	*p* differs between initial capture and subsequent recaptures (where $recap_{i,t}$ is an indicator variable set to 0 until initial capture, 1 after initial capture)
4	M_{plot}	Plot effect	$logit(p_{i,t}) = \beta_0 + \beta_1 * Plot_i$	*p* differs among survey plots (i.e., group effect)
			Models with Continuous Covariates	
6	M_{veg}	Veg. height	$logit(p_{i,t}) = \beta_0 + \beta_1 * veg_i$	*p* depends on vegetation height above the nest entrance
7	M_{hour}	Hour of day	$logit(p_{i,t}) = \beta_0 + \beta_1 * hour_t$	*p* depends on the time of day the survey was conducted
8	M_{temp}	Air temperature	$logit(p_{i,t}) = \beta_0 + \beta_1 * temp_t$	*p* is affected by air temperature during the survey

Figures

Figure 6.1 Representative images of vegetation at study plots (a, b) and *Bombus impatiens* nest entrances (c, d). Only confirmed nests with multiple observations of worker traffic (white arrows) were included in our study.

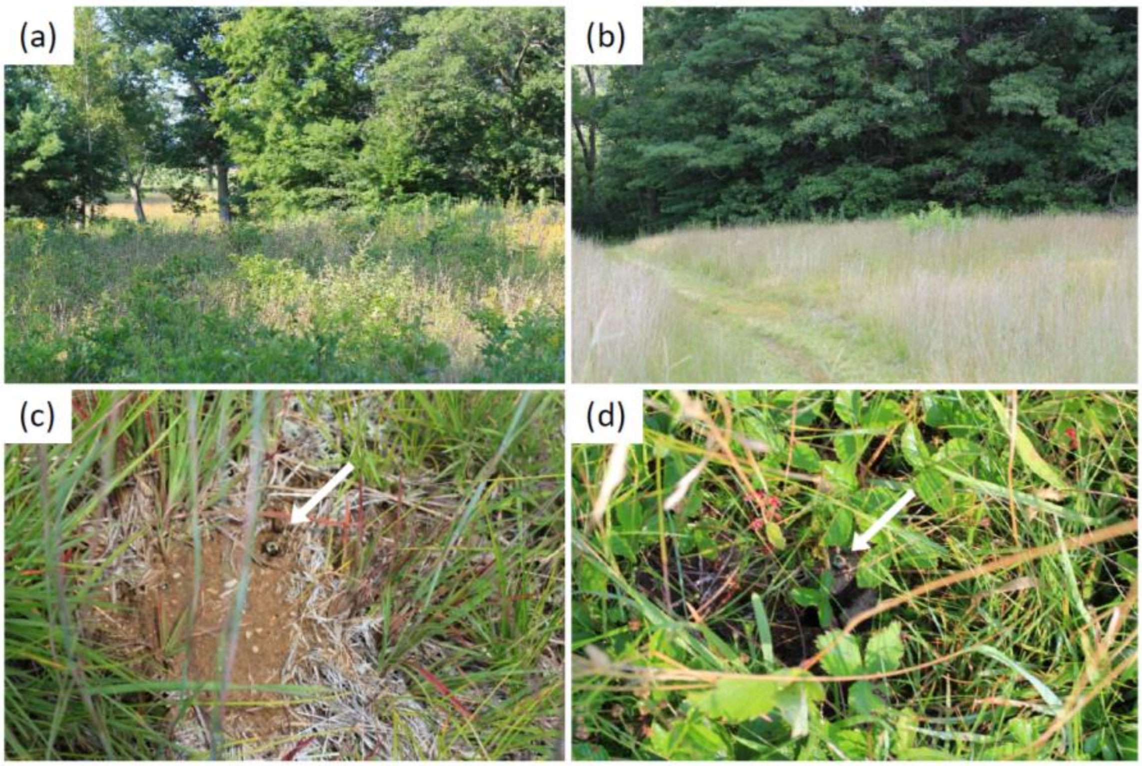


Figure 6.2 Effects on bumble bee nest detection probability from the eight closed population models (n = 18 nests across all plots). Points represent median estimate of effect from Bayesian posterior distributions; lines represent 95% credible intervals.

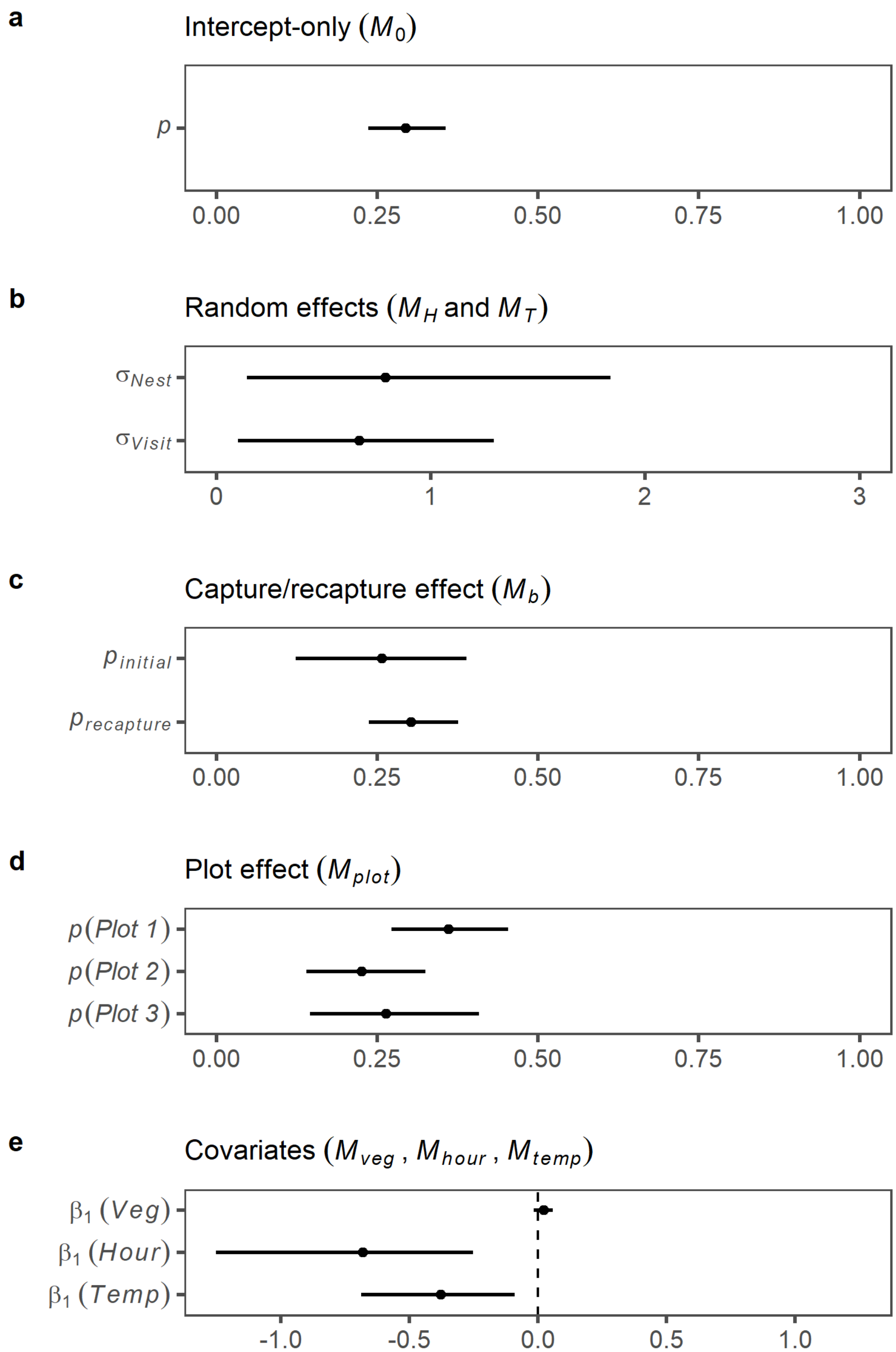


Figure 6.3 Estimated abundance of nests in each of the three survey plots (with associated 95% Bayesian credible intervals) based on each of the eight closed population models and all survey data.

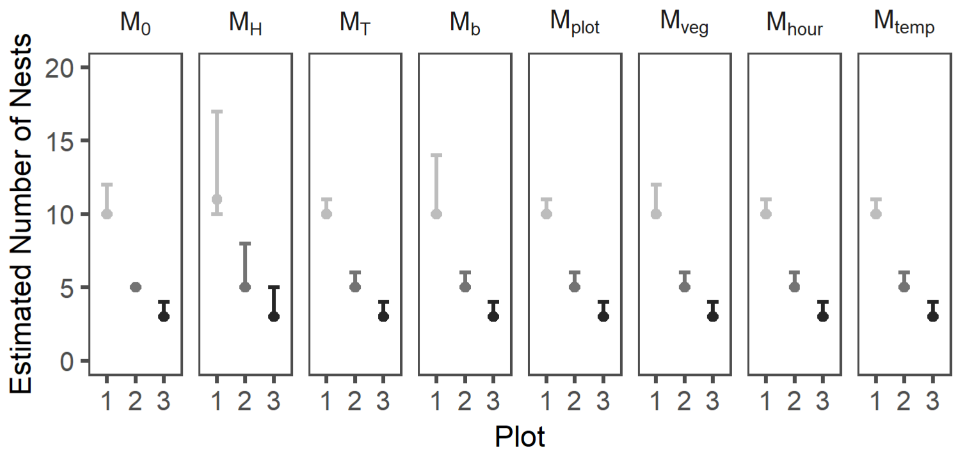
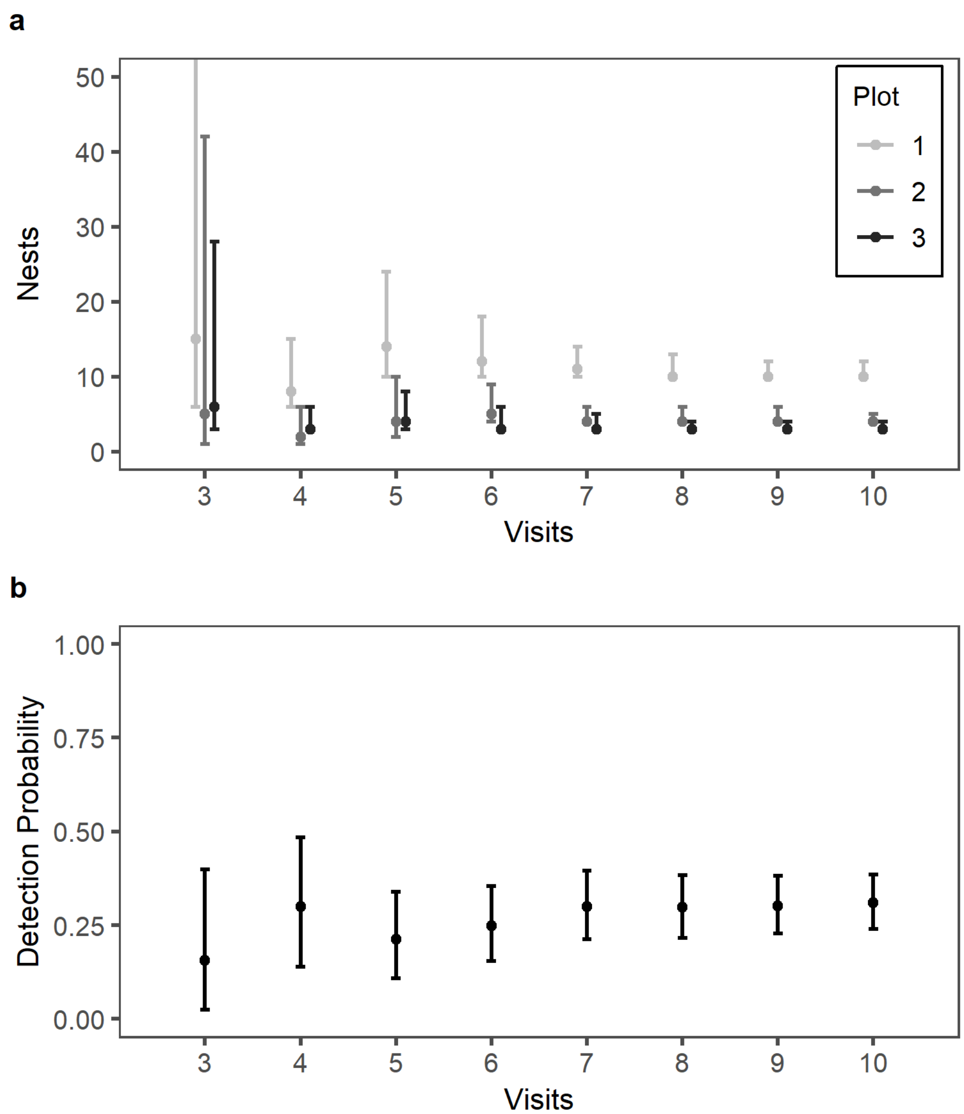


Figure 6.4 Estimated nests in each plot and associated mean detection probability from closed population models, based on model M_0 after different numbers of visits to each plot. Points represent median estimates from Bayesian posterior distribution; lines denote associated 95% credible intervals.



Appendix 6.1

Table S6.1 Summary of previous studies that estimated bumble bee nest density, with description of methods used, study region, species detected, habitat types associated with density estimates, area searched, and estimated density. Note that search area does not apply to molecular methods as nest density is inferred from genetic relatedness between workers and estimates of worker foraging range.

Study	Method	Study Region	Species Detected	Habitat Type	Search area (ha)	Density (nests·ha^{-1})
Osborne et al. 2008b	Fixed Search	UK	Multiple	Grassland <10 cm	0.44	11.4
Osborne et al. 2008b	Fixed Search	UK	Multiple	Grassland >10 cm	0.75	14.6
Osborne et al. 2008b	Fixed Search	UK	Multiple	Woodland	0.19	10.8
Osborne et al. 2008b	Fixed Search	UK	Multiple	Fence line	0.16	37.2
Osborne et al. 2008b	Fixed Search	UK	Multiple	Hedgerow	0.41	29.5
Osborne et al. 2008b	Fixed Search	UK	Multiple	Woodland edge	0.25	19.9
Osborne et al. 2008b	Fixed Search	UK	Multiple	Lrg Garden	0.89	34.9
Osborne et al. 2008b	Fixed Search	UK	Multiple	Med Garden	1.13	31.9
Osborne et al. 2008b	Fixed Search	UK	Multiple	Sm Garden	0.40	50.4
Osborne et al. 2008b	Fixed Search	UK	Multiple	Garden (all)	2.43	35.8
O'Connor et al. 2012	Fixed Search	UK	Multiple	Woodland	0.14	27.8
O'Connor et al. 2012	Detection Dog	UK	Multiple	Woodland	6.94	1.41
O'Connor et al. 2012	Free Search	UK	Multiple	Woodland	6.94	1.44
O'Connor et al. 2017	Free Search	UK	Multiple	Grassland	5.00	3.6
O'Connor et al. 2017	Free Search	UK	Multiple	Woodland	5.00	3

Cumber 1953	Free Search	UK	Multiple	Refuse dump	0.80	48.6
Darvill et al. 2004	Molecular	UK	*B. pascuorum*	Forest/farmland	NA	1.9
Knight et al. 2005	Molecular	UK	*B. pascuorum*	Farmland	NA	0.3
Knight et al. 2009	Molecular	UK	*B. pascuorum*	Farmland	NA	1.7
Darvill et al. 2004	Molecular	UK	*B. terrestris*	Forest/farmland	NA	0.13
Knight et al. 2005	Molecular	UK	*B. terrestris*	Farmland	NA	0.3
Knight et al. 2005	Molecular	UK	*B. lapidarius*	Farmland	NA	1.2
Knight et al. 2005	Molecular	UK	*B. pratorum*	Farmland	NA	0.3
Waters et al. 2010	Sniffer Dog	UK	*B. muscorum*	Upland Heath	NA	0.5
Waters et al. 2010	Sniffer Dog	UK	Multiple	Lowland Heath	NA	0.27
Waters et al. 2010	Sniffer Dog	UK	Multiple	Machair	NA	2.13
Waters et al. 2010	Sniffer Dog	UK	Multiple	Dune	NA	1.47
Rao and Skyrm 2013	Free Search	USA	*B. nevadensis*	Crop field	NA	18.8
Rao and Strange 2012	Molecular	USA	*B. vosnesenskii*	Crop field	NA	0.76
Harder 1986	Free Search	CAN	Multiple	Old field	3.20	10.93

Appendix 6.2

Simulation with two visits to each plot

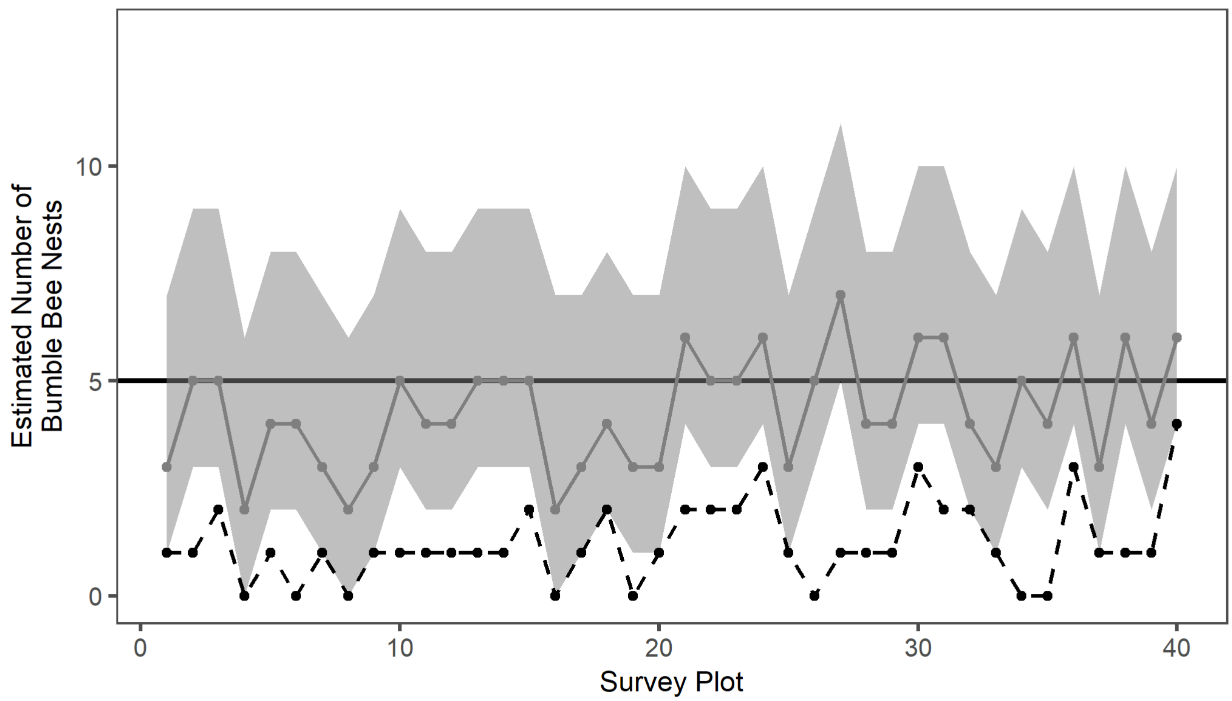
In principal, only two visits are necessary to generate unbiased estimates of nest density at study plots. However, low sample sizes prevented models from converging with only two visits in our empirical study. To confirm that the models we used would converge with only two visits, we conducted a simulation study with a 40 hypothetical plots, each containing exactly 5 bumble bee nests. We used a fixed detection probability of 0.30 for all nests (approximately equal to the mean detection probability we estimated; see *Results* section and Figure 6.2a in main text).

We then simulated two independent visits to each plot. On each visit, each nest in each plot was either detected or not according to a random draw from a Bernoulli distribution with p = 0.3 using the *rbinom(n = 1, size = 1, prob = 0.3)* command in R. The sequence of 0's and 1's for each nest represents its encounter history, where '00' represents a nest that was not detected on either visit, '01' and '10' represent nests that were only detected on a single visit, and '11' represents a nest that was detected on both visits.

Despite the true presence of exactly 5 nests in each of the 40 simulated plots (Figure S6.A1; solid black line), the total number of nests detected in each plot after a single visit ranged from 0 to 4 with a mean of 1.51 and standard deviation across plots of 0.95 (Figure S6.1, dashed line and dots). Thus, there is considerable (but spurious) variation in raw counts of nests across the 40 plots.

We fit mark-recapture model M_0 to the resulting encounter histories, and estimated the number of nests in each plot while accounting for imperfect detection. The model successfully converged with only two visits to each plot and the credible intervals (Figure S6.1; gray ribbons) overlapped the true abundance in every plot. The model M_0 therefore correctly indicates weak evidence for variation in abundance among the 40 simulated plots, despite the high degree of variation in nest counts from a single survey. Thus, for larger sample sizes of plots, two to three visits to each may be sufficient to estimate nest densities.

Figure S6.1 Results of a simulation with 40 survey plots, each with exactly 5 nests, and each nest with a 0.3 probability of detection on a single visit. Black dashed lines and black dots represent counts on a single visit to each plot. Gray lines and dots depict estimates based on a closed population model with 2 visits to each plot. Gray ribbon indicates 95% credible intervals for estimates in each plot. Thick solid line is true number of nests in each plot (n = 5).



Chapter 7: Conclusion

Genevieve Pugesek and Elizabeth E. Crone (2021) Conclusion.

Together, the chapters of my dissertation demonstrate the value and feasibility of locating bumble bee nests directly. By studying naturally occurring bumble bee colonies, we gained valuable insights to the habitat requirements and natural histories of North American *Bombus*. Locating nest sites also provided the opportunity to study the population ecology of the common eastern bumble bee, *B. impatiens*, in further detail.

One of the central goals of my research was to improve our understanding of cryptic life cycle stages, like nesting. In Chapter 2, I characterized species-specific patterns of nesting habitat use for *Bombus* spp., in addition to producing more detailed assessments of habitat use and quality for *B. impatiens*. In Chapter 3, I related patterns of nesting habitat use and quality (observed in Chapter 2) to the movement behavior of nest-searching queens, and found that queen nest-searching strategies were reflective of nest abundance. By highlighting the habitat associations of bumble bees during nest founding, a crucial yet understudied phase of the life cycle, I am hopeful that my research will aid conservation practitioners in their efforts to design effective, practical management guidelines for these pollinators. In addition, as both Chapter 2 and Supplement outline the value of mark-recapture surveys for obtaining accurate estimates of nest density, I hope that these studies will also inform future efforts to monitor bumble bee abundance.

My dissertation research further highlights the value of studying natural bumble bee populations by exploring if studies of commercial/lab-reared bumble bees are representative of bumble bees in the wild. In Chapter 4, I observed behavioral and morphological differences between wild and commercial worker bumble bees, suggesting that commercial bumble bees may be an imperfect substitute for wild bees in studies of foraging ecology. In Chapter 5, I compared field- and lab-based estimates of bumble bee diapause survival, finding that queen *B.*

impatiens in the field had relatively high rates of diapause survival when compared to queens overwintered in the lab. Together, Chapters 4 and 5 emphasize that confirming the ecological relevance of highly controlled experiments is an important step in research, given that these studies are often conducted in isolation from the ecological systems that they represent.

By demonstrating feasible methods for collecting demographic data for wild bumble bee populations, I am hopeful that my research has helped lay the groundwork for future studies of bumble bee population ecology, and that the methods presented here will help bridge the gap between population ecology and habitat associations for bumble bees (and other wild pollinators). Although the methods I have relied upon to complete these studies are fairly time intensive (at least compared to surveys foraging workers), I believe ardently that they are well worth the effort.

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