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Investigating Nest Box Utilization by Bumble Bees and Reproductive Development of Male Bumble Bees

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INVESTIGATING NEST BOX UTILIZATION BY BUMBLE BEES AND REPRODUCTIVE DEVELOPMENT OF MALE BUMBLE BEES

by

James Daniel Herndon

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

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2020

ABSTRACT

INVESTIGATING NEST BOX UTILIZATION BY BUMBLE BEES AND REPRODUCTIVE DEVELOPMENT OF MALE BUMBLE BEES

by

James D. Herndon, Master of Science

Utah State University, 2020

Major Professor: Dr. Karen M. Kapheim
Department: Biology

Bumble bees (*Bombus* Latrielle) provide pollination services which are highly important for food security and ecological resiliency. This thesis centers around two major phases of the bumble bee life cycle. I first investigate nest-founding by wild queen bumble bees in arboreal nest boxes. Given that bumble bees are attracted to blue and UV reflectance, I investigate whether color cue on nest box entrances increases nesting. Since aspect influences floral composition, I compared the frequency of nesting in nest boxes placed on different slope aspects. I compared bumble bee species occurrence in the environment to which species were observed in nest boxes. Slope aspect and color cues of nest boxes did not affect nesting numbers. 34% of nest boxes had queens interact with them, *Bombus appositus* being the most frequent species. This suggests arboreal nest boxes effectively attract specific species like *B. appositus* regardless of placement and color entrance. The second study quantifies changes in the reproductive anatomy of male *Bombus vosnesenskii*. The changes noted were the diminishment of the testes, which day accessory testes were opaque, accessory gland length, and which day trachea concentrated around the testes over a 2-week sampling period. Differences were measured between males from microcolonies and males from queen-right colonies. Major reproductive system changes of *B. vosnesenskii* happened in the first eight days. Changes included a rapid diminishment of testes surface area and accessory teste opacity due to mature sperm content in bees eight days or older. The number of bees with a high concentration of trachea covering the testes surface area increased as males aged while accessory gland length did not increase. Body size was a significant predictor for all metrics of reproductive development. There were no significant differences between microcolony and queen-right colony males. In summary, this research chapter shows that major changes in the reproductive apparatus of *B. vosnesenskii* 8 days post-eclosion and the size of the male affects the male reproductive development, but not colony type. This thesis provides baseline data for understanding bumble bees in their role as effective pollinators by investigating nesting preferences and reproductive development.

(118 pages)

PUBLIC ABSTRACT

INVESTIGATING NEST BOX UTILIZATION BY BUMBLE BEES AND REPRODUCTIVE DEVELOPMENT OF MALE BUMBLE BEES

by

James D. Herndon, Master of Science

Utah State University, 2020

Bumble bees (*Bombus* Latrielle) are a wide-spread group of pollinating insects that are important species to conserve across many environments to ensure both ecological and economic resiliency because they pollinate important agricultural, horticultural, and wild flora. Surprisingly, fundamental questions still remain about this important charismatic group of pollinators. The investigations in this thesis are but two of many topics that require further research. The topics investigated are understanding bumble bee nest site preferences and reproductive development of both sexes of bumble bees. The first experiment (Chapter 2) investigates whether nest boxes elevated off of the ground and attached to trees attract bumble bees to initiate and start nests. This study documents which species interact with these nest boxes the most. Aspect of box placement and blue and ultraviolet (UV) painted entrances are tested for increased nesting by vernal queen bumble bees. Bumble bees interacted with 34% of nest boxes with *Bombus appositus* being the most abundant species observed interacting with nest boxes. Aspect and entrance color showed no significance in increasing nest-box interactions by bumble bee queens. The second experiment (Chapter 3) is the first study to document developmental patterns in the internal reproductive anatomy of adult male bumble bees as they age and investigates differences between males from queenless microcolonies to males produced from standard queenright bumble bee colonies. The species used is a bumble bee of interest for pollination of greenhouse crops, *Bombus vosnesenskii*. Overall, male development continues for up to 7 days in adult males and overall slows down once adult males are 8 days old. No significant differences in development are observed between males from microcolonies and males from queenright colonies but the size of the male is shown to be significant in the reproductive development of male bumble bees. The reproductive development of *B. vosnesenskii* males offers the first insights into development that takes place in bumble bees post-eclosion. These experiments provide knowledge on fundamental questions still unanswered about the bumble bee life cycle. These experiments are much needed to further understand how to utilize and conserve bumble bees for ecological and economic benefit.

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Literature cited:

Pineda, P. M., & Kondratieff, B. C. (2003). Natural History of the Colorado Great Sand Dunes Tiger Beetle, *Cicindela theatina* Rotger *Transactions of the American Entomological Society* (1890-), 129(3), 333–360.https://www.jstor.org/stable/25078819

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CHAPTER 1

INTRODUCTION: PHASES OF THE BUMBLE BEE LIFECYCLE

Thesis overview:

Bumble bees are important pollinators in their native habitats and are now integrated into modern agricultural practices to increase food yields and ensure food security. Even though these species are paramount to wildlands and to food production, many details of their basic biology are still unknown. One of the most effective ways to fill these knowledge gaps is by studying bumble bee colonies in captivity. This thesis investigates periods of the bumble bee life cycle that offer insight into improving bumble bee domestication practices through trap-nesting and captive breeding. Establishing nests using bumble bee nest boxes in wildlands, or trap nesting, provides habitat for wild bumble bees and is a less labor-intensive method of keeping bumble bee colonies. Chapter 2 of this thesis investigates the efficacy of nest boxes elevated off of the ground by attaching them to trees on forested land. Further, I investigate whether mountain slope aspect or entrances painted with colors known to attract bumble bees on nest box entrances increases or decreases the rate of interaction among bumble bees and arboreal nest boxes. While neither mountain slope placement nor painted color entrances on nest boxes increased or decreased nesting events, arboreal nest boxes did have reasonable success in attracting bumble bees, especially *Bombus appositus* Cresson. These results mean that arboreal nest boxes are effective in attracting vernal queens to initiate nests but certain species within a community such as *B. appositus* are more likely to utilize them than other bumble bee species. Chapter 3 is the first study to investigate internal reproductive morphological changes occurring in adult male bumble bees, a major gap in our understanding of bumble bee reproductive biology. Chapter 3 investigates the reproductive maturation of male bumble bees, using a wild species that has been the target of captive breeding programs, *Bombus vosnesenskii* Radoszkowski. Changes measured in adult bees with age include the surface area change in testes as compared to the surface area of the accessory testes; sperm presence in the accessory testes, accessory gland length; and trachea covering most of the surface area of the testes. Using these metrics to quantify morphological change I find that most change in the reproductive apparatus occurs from eclosion and ends at 8 days of age. I also found that while the colony type (microcolony or queen-right colony) doesn't affect the rate of change in these metrics the size of the bee does. This chapter provides the first quantification of the morphological changes occurring in male bumble bees and shows that males of either colony type are similar in morphology and in the changes taking place as they age but larger bees have variation in these morphological variations. Together these research chapters investigate major gaps in the life history of bumble bees. These research chapters show that arboreal nest boxes are effective in trap-nesting some vernal queen bumble bee species and males are still undergoing major developmental processes up to 8 days post eclosion making 8 day old males the earliest age to utilize for mating bumble bees in captivity.

Background:

Humans have been fascinated by and dependent upon bees for millennia (Crittenden, 2011). The earliest fascination with bees came with the exploitation of social bee species that store food in the form of honey. Cave paintings throughout the world from 40,000–8,000 years ago suggest that humans relied on bees as early as the Upper

Paleolithic (Dams & Dams 1977, Crittenden 2011). Marlowe et al. (2014) argue that pre-human hominid species were likely to take energy-rich honey and nutrient–rich larva from wild bee hives, contributing to the caloric investment needed to develop a large brain (Crittenden, 2011; Marlowe et al., 2014). In this way, honey may have been the most important insect related component of the diet of foraging humans of the Pleistocene. The continued advancement of human societies then led to agriculture which then led to the first civilizations. Early civilizations developed apiculture with honey bees in the Old World and meliponiculture with stingless bees in the Neotropics and Australia. Domestication of honey bees provided a reliable source of honey and other goods such as wax and propolis.

Bees still play a vital role in human society, but their utility has shifted from hive products to the agricultural and ecosystem services they provide as pollinators. The primary function of beekeeping in Europe and North America gradually shifted focus from honey production to pollination services to increase certain crop yields of food and seed production in agriculture (Torrey Botanical Society Bulletin 1950; Vogel 1996). Simultaneously, 18th century naturalists became interested in the interactions of insects with wildflower species and gained knowledge on their role in flowering plant reproduction (Vogel 1996). For example, Charles Darwin was fascinated by the flower morphology of orchids and their insect pollinators including bumble bees, then known as humble-bees. These advancements in understanding the benefits of bees to plant reproduction, their role on shaping floral morphology and to shaping larger-scale plant communities has occurred relatively recently. This shifting interest on utility of bees has also expanded out to managing other species of bees that pollinate other plant species differently than honey bees do (James & Pitts-Singer, 2008; Portman et al., 2019). While this paradigm shift brought about much more understanding of the important role that bees play, many fundamental questions still exist today, especially for non-*Apis* species of bees (Cane 1997; Portman et al., 2019). This thesis is a testament to the expanded interest in wild bees and their utility as pollinators, as my goal is to answer some of these questions about non-*Apis* bees.

Today many more bee species have been studied than ever before which has now made some bee species model organisms for understanding many principles in biology such as insect vision (Chittka, 1997; Greiner et al., 2004), gene flow and biogeography (Jackson et al., 2018; Jha & Kremen, 2013; Kapheim et al., 2019; López-Uribe et al., 2016), effects of climate change on insect pollinators (Dew et al., 2019; Miller-Struttmann et al., 2015; Ploquin et al., 2013), the evolution of eusociality (Amsalem et al., 2014; Danforth, 2002; Kapheim et al., 2015; Kapheim et al., 2013; Rehan & Toth, 2015; Shell & Rehan, 2018; Smith et al., 2007; Yanega, 1997), and insect learning and cognition (Ben-shahar & Robinson, 2001; Leonard et al., 2015; Muth et al., 2017; Muth et al., 2015). The disciplines of melittology, pollination biology, and agriculture continue to contribute to our understanding of bees and the plants that they pollinate. While bees are model organisms for these major disciplines in biology, there are still fundamental questions that remain about many species. This thesis focuses on bumble bees (*Bombus*), which play an important role in food production in agricultural settings and ecological processes in wildland landscapes.

Bumble bees are important pollinators of many wild and agricultural plants making them both ecologically and economically valuable (Alford 1975; Goulson 2010;

Potts et al., 2010; Sladen 1912; Strange, 2010). Recent documented declines in bumble bee species due to habitat loss warrants investigations into the habitat needs of bumble bees (Goulson 2010; Potts et al., 2010). Therefore an aim of bumble bee conservation and of this thesis is to provide necessary habitat for bumble bees (Johnson et al., 2019). The other way to bolster their populations while utilizing bumble bees for their pollination is rearing them in captivity or "domesticating" them.

Domestication of bumble bees was first discussed by Sladen (1912) but did not become a commercial industry until the late 1980s for pollination services. Although several bumble bee species have been domesticated, there are primarily two species that are domesticated on an industrial scale; the buff-tailed bumblebee (*B. terrestris*) from Europe and the common eastern bumble bee (*B. impatiens*) from eastern North America. In North America, more than 100,000 domesticated colonies of *B. impatiens* are produced and moved across the continent annually (Strange 2015, Cameron et al 2016). Recent declines in wild bumble bees are associated with pathogen spillover from domesticated bumble bees escaping greenhouses (Cameron et al., 2016; Otterstatter & Thomson, 2008). Further, concerns about domesticated bumble bees naturalizing outside of their native range and affecting native bees include introductions of *B. terrestris* in Japan (Kanbe et al., 2008; Tsuchida et al., 2019), South America (Torretta et al., 2006), Australia (specifically Tasmania) (Hingston et al., 2002; Stout & Goulson, 2000) and deliberate introductions in New Zealand (Macfarlane & Gurr, 1995). Further, *B. impatiens* has been documented outside of its historic range in North America (Looney et al., 2019; Palmier & Sheffield, 2019; Ratti & Colla, 2010), presumably from escaping greenhouse containment. Developing husbandry techniques for locally appropriate bumble bee species that have large colonies and can be industrially produced is one way to eliminate the movement of these non-native species (Strange, 2015).

An emphasis on developing a localized approach of using only regionally appropriate bumble bee species for commercial pollination services would help to eliminate these concerns of hybridization with native species, outcompeting native species, and potential novel pathogen spread from many bumble bees being contained in the same facility (Aizen et al., 2018; Velthuis & van Doorn, 2006). One species considered for commercialization is the Vosnesensky bumble bee (*B. vosnesenskii*) because it is a very common species in its range (Cameron et al., 2011; Strange & Tripodi, 2019), has been shown to be a very effective pollinator for greenhouse tomatoes and cranberries grown in the Pacific Northwest (Broussard et al, 2011; Dogterom et al., 1998; Macfarlane & Gurr, 1995; Strange, 2015). This species is abundant along the Pacific coast of the United States, northern Mexico and southwestern Canada (Koch et al 2012). It produces very large colonies making it an excellent bumble bee species for pollination in greenhouses. Despite interest in this species for greenhouse pollination, some basic knowledge of this species' biology is lacking.

A brief summary of the bumble bee life cycle needs to be understood in order to address which questions about bumble bees remain unanswered. The basic biology of *B. vosnesenskii* (and other bumble bee species) is understood to occur according to the steps listed below as described by Alford (1975), Heinrich (1979), and Goulson (2010):

Life cycle of a bumble bee (example: B. vosnesenskii)

1) Diapause

After mating, the inseminated gyne forages to build up fat to prepare for diapause. Once the gyne has mated she locates an underground hibernaculum and excavates the site to begin diapause. Diapause will last throughout the winter until the average ambient temperature increases to 5-25°C (Heinrich 1979). The queen then breaks diapause and leaves her hibernaculum to begin nesting.

2) Nesting

The gyne exits her hibernaculum, and forages on nectar post-diapause. Once the gyne collects enough nectar for herself, she will then search for a suitable nesting site for her colony. Bumble bee nests are not exposed in the open but are initiated in an enclosed environment, whether it be a cavity or within a clump of dead grass or other surface level dead vegetation. Bumble bees hide their nests by initiating them in the ground, on the ground surface, or sometimes in cavities elevated off of the ground, such as tree holes or bird houses. Bumble bees incubate their brood, so they prefer to find a nest site with some sort of insulation material already present. Old rodent dens are often where many bumble bee species will nest, because rodents insulate their nests and the bumble bees repurpose this insulation. Once the nest site is selected, the gyne then collects pollen to create a provision on which she can oviposit. The gyne secretes wax from her sternal wax glands and forms the wax into a cup (sometimes termed a "honey pot"). She then continues to forage for nectar to make a nectar reserve for the developing colony.

3) Colony development

Once the pollen provision is large enough, the gyne lays a clutch of eggs, coats them in wax she secretes, and incubates them until they hatch into the first larval instar. She progressively provisions the larvae and continues to incubate them as they pupate, and finally eclose as adults. This first clutch of adult bees are workers (sterile females) that take over the tasks of brood care, foraging, and other colony maintenance while the queen increases her egg-laying rate. The colony continues to grow in size and in its number of workers. Colonies of *B. impatiens* and *B. vosnesenskii* typically will produce several hundred workers. Different bumble bee species will invest differently and produce fewer non-reproductive workers. Bumble bees native to the Arctic Circle such as *B. polaris* will lay one clutch of eggs that become workers and immediately afterwards lay reproductive castes (Vogt et al., 1994). This is likely due to the Arctic's truncated flowering season.

4) *Reproductive production*

The colony eventually switches to producing reproductive bees (males, then new gynes). Both *B. impatiens* and *B. vosnesenskii* can produce hundreds of male offspring and dozens of gynes in the wild (Heinrich 1979). *B. impatiens* will produce many new gynes in captivity, but *B. vosnesenskii* does not produce many gynes in captivity. This lack of gyne production has hindered the efforts in creating captive lines of bumble bees.

5) *Mating*

From early summer to late fall, *B. vosnesenskii* gynes (that year's generation of virgin queens) and males leave their natal nests to mate. Gynes typically receive sperm from a single male from a single copulation (Owen & Whidden, 2013). The inseminated gyne does not mate again at any other phase in her life cycle. Mating, like in all social Hymenoptera, is the shortest period of the queen's life cycle, yet immensely impactful in the colony that the gyne will produce the following spring. Unlike in honey bees and stingless bees, males can mate with multiple females and these females enter into a solitary diapause. The cycle then begins again with the inseminated queen going through diapause (Phase 1).

6) *Colony Senescence*

Meanwhile the maternal colony continues producing reproductive bees, but eventually senesces, leaving the only bees to survive through winter being the gynes. In the maternal colony, the queen dies, followed soon after by the workers and the males which are unable to diapause.

Thesis Outline: Focus on phase 2 and 5

This thesis focuses on phase 2 and phase 5 of the bumble bee life cycle. Chapter 2 focuses on Phase 2 in the life cycle of bumble bees by investigating the efficacy of bumble bee nesting in arboreal nest boxes (ANBs) in montane subalpine environments of northern Utah. The geographic placement of ANBs and visual appearance of the entrance of ANBs were manipulated to test whether these variables influence the establishment of bumble bee nests. I tested whether northerly versus south-facing aspect was important on nest selection by placing nests in trees on north and south facing slopes. To test the impact of visual stimuli on nest selection, the entrance was manipulated by painting an ultraviolet blue circle around the nest entrance of half of all nests deployed. Presence of nesting and nesting behaviors were documented to understand the level at which bumble bees established nests in the ANBs. The species of bumble bees in the nest boxes were also recorded to account for whether certain species in the sub-alpine environment had a higher affinity ANBs. This was done by comparing the community of bumble bees known to occur in the environment to the species observed interacting in the ANBs. Other non-*Bombus* species that nested in the boxes were also noted, because they may compete for nesting territory in the nest box and influence the progression of nesting in the ANBs.

Chapter 3 investigates phase 5 of the bumble bee lifecycle by investigating the reproductive development of bumble bees. Specifically, I looked at reproductive development in *B. vosnesenskii* males as they aged by quantifying changes in the surface area of the testes over time relative to the accessory testes, the length of the accessory glands, the presence or absence of sperm in the accessory testes, and the predominance of trachea covering the surface area of the testes. It is often noted in the literature that male insects do not have continual gametogenesis, and that with this limited production of gametes there is a reduction in the internal reproductive apparatus of males (Duchateau & Mariën, 1995; Duvoisin et al., 1999; Ferreira et al., 2004; Tasei et al., 1998). However, there is no literature on the rate of diminishment of the testes as the insect ages. Most studies detail the histological and physiological changes of tissues or germ cells of the testes (Cruz-Landim et al., 1980; Cruz-Landim, 2001), but the study of *B. vosnesenskii* in chapter 3 is intended to quantify morphological changes in the reproductive apparatus as a whole. With these criteria for change in the internal reproductive apparatus, I then make a refined qualitative description of what to expect to observe in the internal reproductive apparatus of bees of various age groups. This may guide captive management protocols in selecting males of the appropriate age for mating.

The following chapters contribute to a better understanding of basic bumble bee biology questions that are still unanswered. Investigating methods of attracting bumble bees to nest in the wild and understanding patterns of reproductive maturation in males in a bumble bee species showing promise to be a future domesticated species are what these chapters entail. I thank you for reading these chapters and hope they are utilized to further understand these important and charismatic insects.

Literature cited:

Aizen, M. A., Smith-Ramírez, C., Morales, C. L., Vieli, L., Sáez, A., Barahona-Segovia, R. M., … Harder, L. D. (2018). Coordinated species importation policies are needed to reduce serious invasions globally: The case of alien bumblebees in South America. *Journal of Applied Ecology*, *56*(1), 100–106. https://doi.org/10.1111/1365-2664.13121

Alford, D. V. (1975). *Bumblebees*. London: Davis-Poynter.

Amsalem, E., Malka, O., Grozinger, C., & Hefetz, A. (2014). Exploring the role of juvenile hormone and vitellogenin in reproduction and social behavior in bumble bees. *BMC Evolutionary Biology*, *14*(1), 45. https://doi.org/10.1186/1471-2148-14-45

Ben-shahar, Y., & Robinson, G. E. (2001). Satiation differentially affects performance in a learning assay by nurse and forager honey bees. *Journal of Comparative Physiology - A Sensory, Neural, and Behavioral Physiology*, *187*(11), 891–899. https://doi.org/10.1007/s00359-001-0260-z

Broussard, M., Rao, S., Stephen, W. P., & White, L. (2011). Native Bees, Honeybees, and Pollination in Oregon Cranberries. *HortScience Horts*, *46*(6), 885–888. Retrieved from https://dx.doi.org/10.21273/hortsci.46.6.885

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., & Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, *108*(2), 662–667. https://doi.org/10.1073/pnas.1014743108

Cameron, Sydney A, Lim, H. C., Lozier, J. D., Duennes, M. A., & Thorp, R. (2016). Test of the invasive pathogen hypothesis of bumble bee decline in North America. *Proceedings of the National Academy of Sciences*, *113*(16), 4386–4391. https://doi.org/10.1073/pnas.1525266113

Cane, J. H. (1997). Ground-nesting bees: The neglected pollinator resource for agriculture. *ISHS Acta Horticulturae*, *437*, 309–324. https://doi.org/10.17660/ActaHortic.1997.437.38

Chittka, L. (1997). Bee color vision is optimal for coding flower color, but flower colors are not optimal for being coded—why? *Israel Journal of Plant Sciences*, *45*(2–3), 115–127. Retrieved from https://dx.doi.org/10.1080/07929978.1997.10676678

Crittenden, A. N. (2011). The Importance of Honey Consumption in Human Evolution. *Food and Foodways*, *19*(4), 257–273. https://doi.org/10.1080/07409710.2011.630618

Cruz-Landim, C., Beig, D., & Silva De Moraes, R. L. M. (1980). The process of differentiation during spermatogenesis in bees (Hymenoptera: Apidae). *Caryologia*, *33*(1), 1–15. https://doi.org/10.1080/00087114.1980.10796814

Cruz-Landim, C. Da. (2001). Organization of the cysts in bee (Hymenoptera: Apidae) testis: Number of spermatozoa per cyst. *Iheringia - Serie Zoologia*, *91*, 183–189. https://doi.org/10.1590/s0073-47212001000200025

Dams, M., & Dams, L. 1977. Spanish rock art depicting honey gathering during the Mesolithic. *Nature*, *268*, 228. Retrieved from http://dx.doi.org/10.1038/268228a0

Danforth, B. N. (2002). Evolution of sociality in a primitively eusocial lineage of bees. *Proceedings of the National Academy of Sciences*, *99*(1), 286–290. https://doi.org/10.1073/pnas.012387999

Dew, R. M., Silva, D. P., Rehan, S. M., Paiva, D., & Rehan, S. M. (2019). Range expansion of an already widespread bee under climate change. *Global Ecology and Conservation*, *17*. https://doi.org/10.1016/j.gecco.2019.e00584

Dogterom, M. H., Matteoni, J. A., & Plowright, R. C. (1998). Pollination of Greenhouse Tomatoes by the North American *Bombus vosnesenskii* (Hymenoptera: Apidae). *Journal of Economic Entomology*, *91*(1), 71–75. https://doi.org/10.1093/jee/91.1.71

Duchateau, M. J., & Mariën, J. (1995). Sexual biology of haploid and diploid males in the bumble bee *Bombus terrestris*. *Insectes Sociaux*, *42*(3), 255–266. https://doi.org/10.1007/BF01240420

Duvoisin, N., Baer, B., & Schmid-Hempel, P. (1999). Sperm transfer and male competition in a bumblebee. *Animal Behaviour*, *58*(4), 743–749. https://doi.org/10.1006/anbe.1999.1196

Ferreira, A., Abdalla, F. C., Kerr, W. E., & Cruz-Landim, C. da. (2004). Comparative anatomy of the male reproductive internal organs of 51 species of bees. *Neotropical Entomology*, *33*(5), 569–576. Retrieved from http://dx.doi.org/10.1590/S1519-566X2004000500005

Goulson, D. 2010. *Bumblebees: Behaviour, ecology, and conservation*. Oxford: Oxford University Press

Greiner, B., Ribi, W. A., & Warrant, E. J. (2004). Retinal and optical adaptations for nocturnal vision in the halictid bee *Megalopta genalis*. *Cell and Tissue Research*, *316*(3), 377–390. https://doi.org/10.1007/s00441-004-0883-9

Heinrich, B. 1979 *Bumblebee Economics*. Harvard University Press, Cambridge, MA and London England

Hingston, A. B., Marsden-Smedley, J., Driscoll, D. A., Corbett, S., Fenton, J., Anderson, R., … Desmarchelier, J. M. (2002). Extent of invasion of Tasmanian native vegetation by the exotic bumblebee *Bombus terrestris* (Apoidea: Apidae). *Austral Ecology*, *27*(2), 162–172. https://doi.org/10.1046/j.1442-9993.2002.01179.x

Jackson, J. M., Pimsler, M. L., Oyen, K. J., Koch-Uhuad, J. B., Herndon, J. D., Strange, J. P., … Lozier, J. D. (2018). Distance, elevation and environment as drivers of diversity and divergence in bumble bees across latitude and altitude. *Molecular Ecology*, *27*(14), 2926–2942. https://doi.org/10.1111/mec.14735

James, R. R., & Pitts-Singer, T. L. (2008). *Bee pollination in agricultural ecosystems*. Oxford University Press.

Jha, S., & Kremen, C. (2013). Urban land use limits regional bumble bee gene flow. *Molecular Ecology*, *22*(9), 2483–2495. https://doi.org/10.1111/mec.12275

Johnson, S. A., Tompkins, M. M., Tompkins, H., & Colla, S. R. (2019). Artificial Domicile Use by Bumble Bees (*Bombus*; Hymenoptera: Apidae) in Ontario, Canada. *Journal of Insect Science*, *19*(7), 1–5. https://doi.org/10.1093/jisesa/iey139

Kanbe, Y., Okada, I., Yoneda, M., Goka, K., & Tsuchida, K. (2008). Interspecific mating of the introduced bumblebee *Bombus terrestris* and the native Japanese bumblebee *Bombus hypocrita* sapporoensis results in inviable hybrids. *Naturwissenschaften*, *95*(10), 1003–1008. https://doi.org/10.1007/s00114-008-0415-7

Kapheim, K. M., Smith, A. R., Nonacs, P., Wcislo, W. T., & Wayne, R. K. (2013). Foundress polyphenism and the origins of eusociality in a facultatively eusocial sweat bee, *Megalopta genalis* (Halictidae). *Behavioral Ecology and Sociobiology*, *67*(2), 331–340. https://doi.org/10.1007/s00265-012-1453-x

Kapheim, K. M., Nonacs, P., Smith, A. R., Wayne, R. K., & Wcislo, W. T. (2015). Kinship, parental manipulation and evolutionary origins of eusociality. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1803), 20142886. https://doi.org/10.1098/rspb.2014.2886

Kapheim, K. M., Pan, H., Cai, L., Blatti III, C. E., Harpur, B. A., Ioannidis, P., Jones, B. M., Kent, C. F., Sloofman, L., Stolle, E., Waterhouse, R. M., Zayed, A., Zhang, G., Wcislo, W. T. (2019) Draft genome assembly and population genetics of an agricultural pollinator, the solitary alkali bee (Halictidae: *Nomia melanderi*). *G3: Genes, Genomes, Genetics*. 9: g3.200865.2018. doi: 10.1534/g3.118.200865

Leonard, A., Muth, F., & Papaj, D. (2015). Colour learning when foraging for nectar and pollen: Bees learn two colours at once. *Biology Letters*. https://doi.org/10.1098/rsbl.2015.0628

Looney, C., Strange, J. P., Freeman, M., Jennings, D., & Jennings, D. (2019). The expanding Pacific Northwest range of *Bombus impatiens* Cresson and its establishment in Washington State. *Biological Invasions*, *21*(6), 1879–1885. https://doi.org/10.1007/s10530-019-01970-6

López-Uribe, M. M., Cane, J. H., Minckley, R. L., & Danforth, B. N. (2016). Crop domestication facilitated rapid geographical expansion of a specialist pollinator, the squash bee *Peponapis pruinosa*. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1833). https://doi.org/10.1098/rspb.2016.0443

Macfarlane, R. P., & Gurr, L. (1995). Distribution of bumble bees in new zealand. *New Zealand Entomologist*, *18*(1), 29–36. https://doi.org/10.1080/00779962.1995.9721999

Marlowe, F. W., Berbesque, J. C., Wood, B., Crittenden, A., Porter, C., & Mabulla, A. (2014). Honey, Hadza, hunter-gatherers, and human evolution. *Journal of Human Evolution*, *71*, 119–128. https://doi.org/10.1016/j.jhevol.2014.03.006

Michener, C. D. (2000). *The Bees of the World*. Baltimore, Md: Johns Hopkins University Press.

Miller-Struttmann, N. E., Geib, J. C., Franklin, J. D., Kevan, P. G., Holdo, R. M., Ebert-May, D., … Galen, C. (2015). Functional mismatch in a bumble bee pollination mutualism under climate change. *Science*, *349*(6255), 1541–1544. https://doi.org/10.1126/science.aab0868

Muth, F., Scampini, A. V, & Leonard, A. S. (2015). The effects of acute stress on learning and memory in bumblebees. *Learning and Motivation*. https://doi.org/10.1016/j.lmot.2014.10.007

Muth, F., Papaj, D. R., & Leonard, A. S. (2017). Multiple rewards have asymmetric effects on learning in bumblebees. *Animal Behaviour*, *126*, 123–133. https://doi.org/10.1016/j.anbehav.2017.01.010

Otterstatter, M. C., & Thomson, J. D. (2008). Does Pathogen Spillover from Commercially Reared Bumble Bees Threaten Wild Pollinators? *PLOS ONE*, *3*(7), 1–9. https://doi.org/10.1371/journal.pone.0002771

Owen, R. E., & Whidden, T. L. (2013). Monandry and polyandry in three species of North American bumble bees (Bombus) determined using microsatellite DNA markers. *Canadian Journal of Zoology*, *91*(7), 523–528. https://doi.org/10.1139/cjz-2012-0288

Palmier, K., & Sheffield, C. (2019). First records of the Common Eastern Bumble Bee, Bombus impatiens Cresson (Hymenoptera: Apidae, Apinae, Bombini) from the Prairies Ecozone in Canada. *Biodiversity Data Journal*, *7*, e30953. https://doi.org/10.3897/bdj.7.e30953

Ploquin, E. F., Herrera, J. M., & Obeso, J. R. (2013). Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia* 173(4) 1649–1660. https://doi.org/10.1007/s00442-013-2731-7

Portman, Z. M., Orr, M. C., & Griswold, T. (2019). A review and updated classification of pollen gathering behavior in bees (Hymenoptera, Apoidea). *Journal of Hymenoptera Research*, *71*, 171–208. https://doi.org/10.3897/jhr.71.32671

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Ratti, C. M., & Colla, S. R. (2010). Discussion of the presence of an eastern bumble bee species (*Bombus impatiens* Cresson) in western Canada. *The Pan-Pacific Entomologist*, *86*(2), 29–31. https://doi.org/10.3956/2009-19.1

Rehan, S. M., & Toth, A. L. (2015). Climbing the social ladder: The molecular evolution of sociality. *Trends in Ecology & Evolution*, *30*(7), 426–433. https://doi.org/10.1016/j.tree.2015.05.004

Shell, W. A., & Rehan, S. M. (2018). Behavioral and genetic mechanisms of social evolution: insights from incipiently and facultatively social bees. *Apidologie*, *49*(1), 13–30. https://doi.org/10.1007/s13592-017-0527-1

Smith, A. R., Wcislo, W. T., & O'Donnell, S. (2007). Survival and productivity benefits to social nesting in the sweat bee *Megalopta genalis* (Hymenoptera: Halictidae). *Behavioral Ecology and Sociobiology*, *61*(7), 1111–1120. https://doi.org/10.1007/s00265-006-0344-4

Stout, J. C., & Goulson, D. (2000). Bumble bees in Tasmania: their distribution and potential impact on Australian flora and fauna. *Bee World*, *81*(2), 80–86. https://doi.org/10.1080/0005772X.2000.11099475

Strange, J. P. (2010). Nest Initiation in Three North American Bumble Bees (*Bombus*): Gyne Number and Presence of Honey Bee Workers Influence Establishment Success and Colony Size. *Journal of Insect Science*, *10*(130), 1–11. https://doi.org/10.1673/031.010.13001

Strange, J. P. (2015). *Bombus huntii*, *Bombus impatiens*, and *Bombus vosnesenskii* (Hymenoptera: Apidae) Pollinate Greenhouse-Grown Tomatoes in Western North America. *Journal of Economic Entomology*, *108*(3), 873–879. https://doi.org/10.1093/jee/tov078

Strange, J. P., & Tripodi, A. D. (2019). Characterizing bumble bee (*Bombus*) communities in the United States and assessing a conservation monitoring method. *Ecology and Evolution*, *9*(3), 1061–1069. https://doi.org/10.1002/ece3.4783

Tasei, J. N., Moinard, C., Moreau, L., Himpens, B., & Guyonnaud, S. (1998). Relationship between aging, mating and sperm production in captive *Bombus terrestris*. *Journal of Apicultural Research*, *37*(2), 107–113. https://doi.org/10.1080/00218839.1998.11100962

Torretta, J. P., Medan, D., & Abrahamovich, A. H. (2006). First record of the invasive bumblebee *Bombus terrestris* (L.) (Hymenoptera, Apidae) in Argentina. *Transactions of the American Entomological Society*, *132*(3), 285–289. https://doi.org/10.3157/0002-8320(2006)132[285:frotib]2.0.co;2

Tsuchida, K., Yamaguchi, A., Kanbe, Y., & Goka, K. (2019). Reproductive interference in an introduced bumblebee: Polyandry may mitigate negative reproductive impact. *Insects*, Vol. 10, p. 59. https://doi.org/10.3390/insects10020059

Velthuis, H. H. W. W., & van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, *37*(4), 421–451. https://doi.org/10.1051/apido:2006019

Vogel, S. 1996: Christian Konrad Sprengel's Theory of the Flower: The Cradle of Floral Ecology. *Floral Biology: Studies on Floral Evolution in animal-Pollinated Plants*. Chapman & Hall, New York.

Vogt, F. D., Heinrich, B., Dabolt, T. O., & McBath, H. L. (1994). Ovary development and colony founding in subarctic and temperature-zone bumblebee queens. *Canadian Journal of Zoology*, *72*(9), 1551–1556. https://doi.org/10.1139/z94-206

Yanega, D. (1997). Demography and sociality in halictine bees (Hymenoptera: Halictidae). In *The Evolution of Social Behavior in Insects and Arachnids* (pp. 293–315). https://doi.org/10.1017/cbo9780511721953.015

CHAPTER 2

BUMBLE BEE ARBOREAL NEST BOX EFFICACY IN A MONTANE SUBALPINE ENVIRONMENT

Abstract:

Bumble bees (*Bombus* spp.) are key pollinators across many habitats, but several species within this group are experiencing dramatic declines. Insights into bumble bee nest establishment are essential in managing and conserving these important pollinators. Establishing colonies in lab settings is time-consuming, costly, and variable in success rate. Providing proper nesting habitat to establish colonies in the field would save time, effort, money, and may reduce pathogen exposure from captive breeding programs. Self-established colonies can also provide researchers with access to observations on wild colony initiation, growth, and symbiotic relationships among *Bombus* and other species. A potential way to increase wild colony abundance is to increase nesting habitat by providing arboreal nest boxes for wild queens to establish colonies in the wild. I investigated bumble bee use of arboreal nest boxes as a function of landscape attributes and visual stimuli in montane subalpine habitat in northern Utah, USA over two years. Of the 204 nest boxes sampled, 34% showed some sign of *Bombus* activity and 16% were occupied by colonies that produced workers. Aspect placement and visual color cue did not significantly affect the likelihood of *Bombus* activity in the box, but boxes on south facing slopes without color cues tended to have the highest levels of bumble bees interacting with them. We observed the presence of the social parasite *B. insularis* and nesting from other non-focal species that may compete for nest-sites with bumble bees.

This study suggests that arboreal nest boxes may be an effective means of encouraging bumble bee nesting in natural landscapes.

Keywords: *Bombus appositus*, Vespinae, Aspect, Artificial domiciles, *Troglodytes pacificus*, nest usurpation, trap-nesting, social parasitism

Introduction:

It is critical to conserve bumble bees because they are abundant and adaptable pollinators in a wide variety of ecosystems (Goulson 2010; Velthuis & van Doorn 2006). Bumble bees are generalist pollinators that pollinate both common and rare plants, so conserving them in natural environments helps to conserve a wide variety of plant species that many other organisms depend on (Ackerman, 1981; Pitts-Singeret al., 2002; Rhoades et al., 2016; Tuomi, et al., 2015). Bumble bees colonies are composed of many individual bees with subsequent castes (queens, workers, and males) providing robust pollination services over a wide temporal range (Alford 1975; Goulson 2010; Heinrich 1979; Ostevik et al. 2010). The reproductive castes have differing, behavior, foraging and dispersal patterns (Ostevik et al., 2010) The predominant castes pollinating wild flowers are the workers but both queens and males are also pollinators visiting flowers for vital resources in early spring and in late summer/fall. Vernal queens forage flowers to increase metabolic activity and to collect pollen as nesting substrate while in the fall new gynes are foraging plants for building fat body for diapause and eventually ovary development to produce eggs and males are foraging nectar and establishing territory to mate with a new gyne (Ackerman 1981; Alford 1975; Goulson 2010; Heinrich 1979; Malfi et al., 2019; Martinet et al., 2019; Ostevik et al. 2010; Vogt et al., 1998).For these reasons, conserving bumble bees is of utmost importance in broader landscape scale conservation efforts (Williams & Osborne, 2009).

Many factors affect bumble bee communities, including the absence of proper nesting habitat due to various types of land management (Goulson 2010). Anthropogenic stressors include land-management practices such as urban development without proper greenspaces, intensified conventional agriculture, and high frequency grazing regimes (Goulson 2010). These types of land-usage can be deleterious to bees finding proper nesting habitat because they alter soil composition for bumble bees that nest in burrows (Potts et al., 2010).

A common approach to bolster habitat for bumble bee conservation is enhancement of floral resources within otherwise depleted habitats, such as agricultural or urban environments (Cameron et al., 2011; Cusser & Goodell, 2013; Jacobson et al., 2018; Malfi et al., 2019; Martinet et al., 2019; Pywell, 2005; Spivak et al., 2011). While these efforts have been successful, this does not address nesting availability, which is also a critical aspect of bumble bee conservation (Johnson et al., 2019; Lye et al. 2011; Potts et al., 2010; Spivak et al., 2011). Utilizing nest boxes is an additional component in efforts to conserve these pollinators by bolstering habitat availability for them. Some success has been shown in establishing bumble bees inside of nest boxes by simply placing them in a landscape containing bumble bees. Typically managing for both habitat and foraging preferences is a more holistic approach to conserving any species of interest.

Man-made nesting structures for the purpose of animal nesting have been used to conserve and increase populations of various organisms where the environment may lack the nesting requirements of that organism (Baranauskas, 2009; Bender et al., 2016; Bortolotti, 1994; Radunzel et al., 1997). While this approach has been previously implemented in bee conservation efforts, the results have been mixed for bumble bees (Johnson et al. 2019; Sladen 1912; Frison 1926; Fye & Medlar 1954). Nest boxes of many different designs have been built and deployed in a variety of ways to attract bumble bees to nest in them (Donovan and Wier 2012; Fye and Medler 1954; Hobbs et al 1960; Johnson et al., 2019; Lye et al. 2011). These studies started in 1912 with Sladen and have been conducted across North America, Europe, and New Zealand (all non-native species) with highly varied results ranging from 0% to over 50% of nest boxes deployed containing bumble bee colonies (Donovan and Wier 2012; Frison 1926; Fye and Medler 1954; Hobbs, et al., 1960; Johnson et al., 2019; Lye et al. 2011; Sladen 1912). In North America, 21 species of bumble bees have been shown to use nest boxes, including species that face population declines (Johnson et al., 2019). Many of these studies on creating nesting structures have mixed results due to variation in experimental design specifically in design and materials of nest boxes. Barron et al., 2000; Donovan & Wier, 1978; Fye & Medler, 1954; Hobbs et al., 1960; Johnson et al., 2019; Lye et al., 2011). Further investigation of what types of structures bumble bees prefer to nest in need further investigation as these studies provide tools for land managers in conserving these important insect pollinators. This study follows up on some previous work by Dr. James P. Strange where nest boxes were deployed within a subalpine montane portion of the Cache-Wasatch National Forest known as Tony Grove. Design of this experiment was informed by observations and preliminary data of the nest boxes placed there in 2009.

Previous studies have deployed boxes that are buried, placed on the ground surface, or suspended off of the ground (Donovan & Wier 2012; Fye & Medler 1954; Hobbs et al., 1960; Johnson et al., 2019; Lye et al., 2011; Sladen 1912). A recent comparison of these three placements of nest boxes demonstrated that boxes suspended off of the ground have more bumble bees nest in them (Johnson et al., 2019). Additionally, prior observations by Strange (unpublished data) where nest boxes were mostly subterranean or at surface level above-ground showed 16.3% of nest boxes showed evidence of bumble bees interacting with nest boxes, but only 1.9 % developed into bumble bee colonies (Strange unpublished data). A small subsample of nest boxes in 2009 were placed above ground on the sides of tree trunks. Arboreal nest boxes (ANBs) were the smallest sample of boxes (n = 5), but 40% contained fully developed bumble bee nests by the white-shouldered bumble bee (*B. appositus*) (Strange, unpublished data). In Europe and North America there are bumble bees known to nest in tree cavities and bird boxes. The tree bumblebee (*B. hypnorum*) is a known tree nesting species, preferring to nest above ground more than other bumble bees that co-occur in this species' habitat (Crowther et al., 2014) and there are North American species that have been shown to nest in ANBs (Hobbs et al., 1960). Given the past success of ANBs in other studies I used only ANBs in this study. I tested whether the placement of ANBs on a given aspect and color cues on the nest box entrance would increase or decrease bumble bee nesting in the ANBs.

A challenge in having wild bumble bees establish in ANBs is attracting foundress queens to the boxes. There is very little known about what attracts a bumble bee queen to a nesting site and how bumble bee queens find these nesting sites (Goulson 2003;

Richards 1978; Goulson 2010). Because of this lack of understanding of nest site initiation preference, most studies have mimicked characteristics known from natural nesting sites but with well-developed colonies and not in the initiation phase of the colony cycle. Abandoned rodent dens often make ideal nesting habitat for bumble bees (Alford 1975; Sladen 1912). Rodents excavate a cavity and collect some type of insulation material to line that cavity to start nesting. Bumble bees require, but do not collect, insulating material for nests. They therefore utilize left over nesting material from rodent nests to insulate incubating brood and the colony as a whole (Goulson 2010; Velthuis & van Doorn, 2006). This known nesting preference can be emulated by creating an enclosure/cavity with an enclosure with some kind of insulation material (Alford 1975; Goulson 2010; Sladen 1912). Emulating abandoned rodent nests by placing insulation material has shown success in getting bumble bees nest in artificial domiciles (Alford 1975; Sladen 1912).

Other attempts to increase nesting in nest boxes have taken further steps to emulate old rodent dens through bumble bee foundress sensory cues such as olfaction by placing upholsters cotton that rodents had used for nesting inside of the nest structure (Slatkosky, personal communication). However, these efforts have shown little to no success to increase nesting in bumble bee nest boxes. However, there may be other sensory cues produced from rodents that bumble bees use to detect nesting sites.

Visual cues may attract bumble bees to ANBs and in turn increase nesting in ANBs with added visual stimuli on the ANB entrance. The visual system is a major component of the bumble bee neural anatomy and has a significant impact on many aspects of the bumble bee's interaction with its environment (Raine and Chittka 2005).

Specifically, bumble bees have a high visual acuity to blue wavelengths and also are able to visualize ultraviolet (UV) wavelengths (Raine and Chittka 2005). The ability to detect UV reflectance aids in many functions across insect taxa including hunting/foraging, sexual signaling, and nest site recognition (Honkavaara et al., 2002). Bumble bees may find rodent nests to uptake as their own through other sensory stimuli and one such stimulus may be the abundance of UV reflectance around rodent nests. Rodent urine contains high concentrations of the highly UV reflective compound uric acid so rodent nests and rodent trails give off a lot of UV reflectance (Honkavaara et al., 2002; Viitala et al., 1995). Raptorial birds which also are able to detect UV reflectance visually, use this uric acid reflectance to stalk and hunt rodents (Honkavaara et al., 2002; Viitala et al., 1995). It is therefore possible that bumble bees may also use UV reflectance from rodents to identify old rodent nests sites. Bumble bees also show an attraction to blue UV before learning which colors are associated with food rewards as evidenced by behavioral experiments (Raine & Chittka, 2005) and in passive sampling protocols using blue vane traps (Sircom et al., 2018). I tested the hypothesis that bumble bee use of ANBs would increase in response to a visual stimulus of blue UV paint around the entrance.

Aspect is a physical landscape characteristic that may influence nest site preferences of bumble bees and affect the efficacy of ANBs. Landscape features have been shown to affect bumble bee abundance and diversity in montane environments (Hatfield & Lebuhn, 2007). It has been well documented that aspect (slope direction of a landscape) has profound effects on floral composition due to different rates of snowmelt because of differing sun exposure on the cardinal direction the slope faces (Gillott 2003). In the northern hemisphere, south-facing slopes have a higher rate of snow melt than northerly slopes (Billings & Bliss, 1959; Jones et al., 1985). The effect of aspect difference is particularly pronounced at high elevations (Billings & Bliss, 1959; Jones et al., 1985). Figure 2-1 is exemplary of many regions throughout the subalpine zone of the mountain western U.S., south-facing slopes are xeric with a mix of mountain-brush and aspen forest edge environments, while northerly slopes retain more moisture and thus have a continual canopy cover from a mixed conifer forest with patches of aspen. Both snow melt and angiosperm community differences on these slopes likely affect when vernal bumble bee queens break diapause and where they search for nest sites. Bumble bees in diapause on south facing slopes likely break diapause sooner, and are therefore seeking nest sites sooner than bumble bees in diapause on north-facing slopes (Heinrich, 1984; Vogt et al., 1994). The only natural nest sites available for bumble bees that nest in the ground would be on south-facing slopes. Arboreal nest boxes may provide earlier habitat for the earliest emerging bees but temperature differences and floral foraging resources differ considerably between slopes. Given the proximity to the earliest vernal queens, earlier flower phenology, and warmer temperature of south facing slopes it is most likely for bees to nest in south-facing ANBs than in northerly ANBs.

Northern Utah’s rich bumble bee fauna offers an opportunity to test which of a wide variety of species are attracted to ANBs. I focused on a small basin called Tony Grove. This USEPA Level III ecoregion, the Wasatch and Uinta Mountains, has a total of 18 species historically and in a recent survey (Strange & Tripodi 2019) 12 species were detected at Tony Grove. This species richness allows for us to investigate the efficacy of ANBs across many different bumble bee species. This also offers unique insights into how species interact as they initiate and develop colonies in nature.

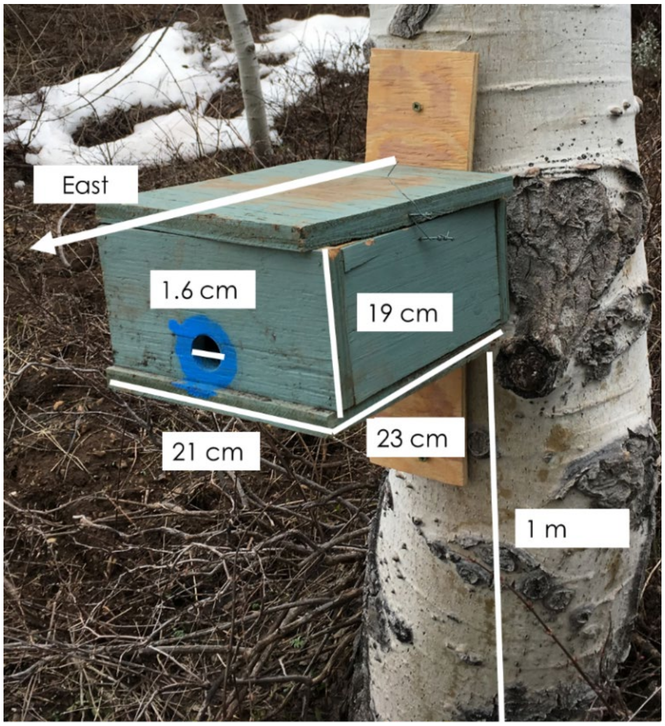
Figure 2-1. Photo from mid-June 2016 illustrating differences in flora due to aspect. This photo was from a south-facing slope and the north facing slope is visible in the distance to the left of the road system in this valley bottom. This photo shows that while south-facing slope flora is dominated by shrubs and largely dry open meadow and aspen woodland north facing slopes have a mixed conifer forest. This photo also shows differences in phenology of the two slopes where the south-facing slope has many vernal plants in bloom while there are still patches of snow on the north-facing slope.

Methods:

Nest box construction and installation

Bumble bee nest boxes were constructed using 12.5 mm plywood (23 x 21 x 19 cm with a 1.6 cm diameter entrance) and installed above ground on trees at Tony Grove (N 41.888193, W 111.603520) in the Uinta-Wasatch-Cache National Forest before spring bloom and complete snow melt in the last week of May. Upholsters cotton was added to each box as a nest insulation substrate (Hobbs et al, 1960; Johnson et al., 2019; Plowright & Jay 1966). All nest entrances were facing an easterly cardinal direction. Half of the boxes had a blue UV reflective paint around the entrance. I placed 102 boxes in clusters of groups of six. There was a total of seventeen clusters, with nine clusters placed on north-facing slopes and eight clusters on south-facing slopes. In each cluster of six nest boxes, three of the nest boxes had blue/UV reflectant paint around the entrance (Figure 2-2) and three boxes did not have a color cue on the nest entrance. I performed the study in 2016 and 2017 for a total of 204 ANBs. Nest boxes were left up between years and repaired as needed in 2017 to begin that year's census of ANBs.

A)



B)

Figure 2-2.A) An example of an ANB installed on a south-facing slope with a blue/UV painted entrance showing dimensions of the ANB. Upholsters cotton was placed inside of

each box. B) Installation of ANB on north facing slope. Both photos were taken May 2016. Note the difference in snow level in A) compared with B)

Nest Box Censuses

Censuses of all ANBs were conducted once every two weeks after installation. At each census, we searched the contents of the ANBs for evidence of bumble bees and recorded 3 developmental categories of bumble bee occupancy: 1) “none” meaning there was no evidence of bumble bees interacting with the box; 2) “nest initiation” which encompassed observing a queen in the box or any progressive behavior in nest building up to the point of having brood present; and 3) “social phase” when workers were present in the colony. These categories differentiated how many boxes bumble bees interacted with and how many boxes actually contained mature colonies that may have produced gynes and males. Specific observations and how they were categorized are listed in Table 2-1. When possible, the bumble bee species present in the box was recorded.

To investigate whether some species prefer nest boxes more than other species, the abundance and diversity of bees occupying nest boxes were compared to community data recorded in Tony Grove (Strange and Tripodi 2019). In mid-September, all contents of every ANB were collected and searched for evidence of bumble bees that were missed during field checks. Observations of any *Psithyrus* species were noted as well as any other animal found nesting within the ANBs.

Table 2-1

Observations recorded for evidence of bumble bees. The right column shows the categorization of the evidence of bumble bees in the nest box.

Evidence of bee	Description	Developmental Category
None	No evidence of bumble bee presence	No nesting
Bee entered box	Bee was seen inside the box or observed entering the box	Nest initiation
Pollen present	Pollen was found in the cotton substrate with or without a bee present.	
Wax honey pot present	Wax was found in the cotton substrate with or without a bee present.	
Brood present	Brood was found on a pollen provision with or without the presence of a bumble bee.	
Workers present	Workers present within the box. Species of workers was also documented.	Social phase

Statistical Analysis

Logistic regression was used to compare year of survey, the presence or absence of a blue/UV color entrance, and aspect (north versus south facing slope) for all boxes. The binomial distribution for this model was boxes with no interaction from bumble bees and boxes with any type of interaction by bumble bees, regardless of whether they reached the social phase or not (n = 204). Analysis was conducted in R version 3.6.1 using the “glm” function in the lme4 package (Bates, Mächler, Bolker, & Walker, 2015). A second logistic regression was run on only boxes that had any sign of bumble bees (n = 70). The same variables from the previous model were used in this model (year, presence or absence of a blue/UV color entrance, aspect, and aspect in relation to year), but the binomial distribution was amongst nest boxes that reached social phase or boxes with any other sign of bumble bee interaction. A probability table was made for each unique condition to find which unique scenario with the ANBs had the highest bumble bee interaction. A table of the 8 unique conditions can be found in Table 2-2 below. Finally, a chi square analysis was used to compare the community of bumble bees using ANBs compared to bumble bee community surveyed by net collection on flowers in area (Strange & Tripodi, 2019).

Table 2-2.

List of unique conditions ANBs were placed in

Unique conditions	Year	Blue/UV color cue present	Aspect	Sample size (n)
1	2016	No	North	27
2	2016	Yes	North	27
3	2016	No	South	24
4	2016	Yes	South	24
5	2017	No	North	27
6	2017	Yes	North	27
7	2017	No	South	24
8	2017	Yes	South	24

Results:

Bumble bees interacted with 34% and achieved social phase in 16% of all ANBs

Over the two-year survey, 34.32% (n = 70 nest boxes) of nest boxes had recorded bumble bee activity (Table 2). Of these 70 ANBs, 18.14% (n = 37) did not progress beyond nest initiation and 16.18% (n = 33) eventually had colonies that developed into the social phase (Table 2-3).

Six species of bumble bees detected in ANBs

Six species of *Bombus* were detected in the nest boxes, 5 of the species detected were non-parasitic *Bombus* and one was in the parasitic subgenus *Psithyrus*. *B. appositus* was the species most frequently (n = 27) encountered and the most likely to advance to social phase in ANBs (n = 22) (Table 2-3) All *B. appositus* nests that reached social phase had one or more conspecific queen found dead inside the ANB.

Table 2-3

All observations of bumble bees interacting with the arboreal nest boxes. Observations were categorized as either nest initiation or social phase. All species documented are listed by row. Total *Bombus* row is the sum of all of the bumble bee species observed. Unknown *Bombus* represent nests where evidence of nesting occurred, but no adult bees were found to allow for species identification. *Psithyrus* is not in this table because they usurp other bumble bee colonies and never initiate nests.

Aerial Nest Boxes with *Bombus* interactions			
Bumble bee species	Nest Initiation	Social Phase	Sum of interactions
B. appositus	5	22	27
B. centralis	1	4	5
B. huntii	1	3	4
B. mixtus	0	3	3
B. rufocinctus	2	1	3
Unknown *Bombus*	28	N/A	28
Total *Bombus*	37	33	70

Year, aspect and nest entrance color did not predict whether bumble bees would interact with ANBs

Year ($z = 1.42$ $p = 0.156$), aspect ($z = 1.138$ $p = 0.255$), and entrance color ($z = 1.478$ $p = 0.139$) showed no significance in predicting the number of boxes that had bumble bees interact with them ($\chi^2 = 4.2729$; $N = 204$; $p = 0.3703$) (See tables 2-4, 2-5, 2-6). Year ($z = -0.12$ $p = 0.904$), aspect ($z = 1.14$ $p = 0.255$), and color cue on the nest entrance ($z = 0.56$ $p = 0.575$) also showed no significance in predicting which boxes would develop into a social phase colony ($\chi^2 = 6.4136$; $N = 70$; $p = 0.1703$). Table 2-7 lists the scenarios and probability of nesting based on the results of the survey. Boxes installed on a southern aspect without a blue/UV color cue in 2016 were the most likely boxes to have bumble bees interact with them in this experiment.

Table 2-4

All observations of bumble bees interacting with the arboreal nest boxes. Observations were categorized as either nest initiation or social phase. All species documented are listed by row. Total *Bombus* row is the sum of all of the bumble bee species observed.

Comparisons between year of survey: 2016 and 2017							
	Nest Initiation			Social Phase			Sum of all *Bombus* interactions
Bumble bee species	2016	2017	All nest boxes	2016	2017	All nest boxes	
B. appositus	4	1	5	10	12	22	27
B. centralis	1	0	1	3	1	4	5
B. huntii	0	1	1	2	1	3	4
B. mixtus	0	0	0	0	3	3	3
B. rufocinctus	2	0	2	1	0	1	3
Unknown *Bombus*	15	13	28	N/A	N/A	N/A	28
Total *Bombus*	22	15	37	16	17	33	70

Table 2-5

The number of boxes that initiated nests and how many made it to the social phase for each aspect placement. All species documented are listed by row. Total *Bombus* row is the sum of all of the bumble bee species observed

Aspect: Boxes placed on North vs. South facing slopes							
	Nest Initiation			Social Phase			Sum of all *Bombus* interactions
Bumble bee species	North	South	All nest boxes	North	South	All nest boxes	
B. appositus	4	1	5	8	14	22	27
B. centralis	1	0	1	2	2	4	5
B. huntii	0	1	1	2	1	3	4
B. mixtus	0	0	0	0	3	3	3
B. rufocinctus	0	2	2	1	0	1	3
unknown *Bombus*	19	9	28	N/A	N/A	N/A	28
Total *Bombus*	24	13	37	13	20	33	70

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Table 2-6

The number of boxes that initiated nests and made it to the social phase for each aspect placement. All species documented are listed by row. Total *Bombus* row is the sum of all of the bumble bee species observed.

Presence of Blue/UV color cue: No Color vs. Blue/UV Entrance							
	Nest initiation			Social phase			Sum of all *Bombus* interactions
Bumble bee species	No color	Blue/UV entrance	Total	No color	Blue/UV entrance	Total	
B. appositus	1	4	5	13	9	22	27
B. centralis	0	1	1	3	1	4	5
B. huntii	1	0	1	1	2	3	4
B. mixtus	0	0	0	0	3	3	3
B. rufocinctus	1	1	2	1	0	1	3
unknown *Bombus*	19	9	28	N/A	N/A	N/A	28
Total *Bombus*	22	15	36	18	15	33	70

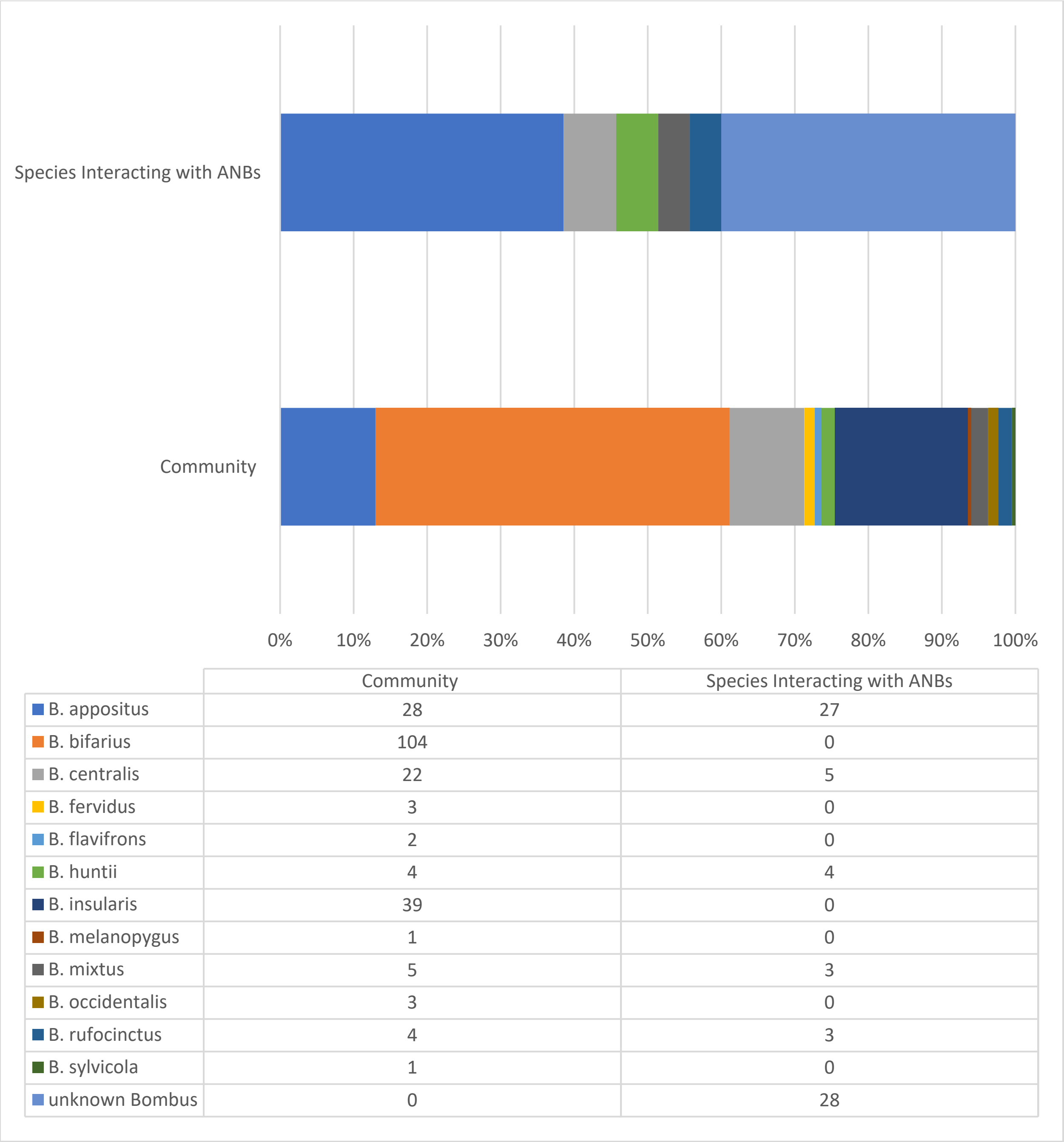
Table 2-7

Probability table of each unique scenario of the nest boxes. The boxes with the highest success were nest boxes placed on a south facing slope without a blue/UV entrance in 2016.

Unique conditions	Year	Blue/UV color cue present	Aspect	No *Bombus*	Nest Initiation observed	Social phase	Total observations of *Bombus*
1	2016	No	North	60.35%	20.26%	19.39%	39.65%
2	2016	Yes	North	69.35%	16.72%	13.93%	30.65%
3	2016	No	South	57.32%	21.26%	21.42%	42.68%
4	2016	Yes	South	66.62%	17.88%	15.50%	33.38%
5	2017	No	North	65.06%	18.51%	16.43%	34.94%
6	2017	Yes	North	73.45%	14.86%	11.69%	26.55%
7	2017	No	South	62.15%	19.62%	18.23%	37.85%
8	2017	Yes	South	70.93%	16.02%	13.04%	29.07%

Bumble bee species interacting with ANBs were not exemplary of the bumble bee community.

B. appositus was the most common species found interacting with the ANBs (n = 27; Fig. 2-3). However, this species is not the most common species of bumble bee found at Tony Grove (Fig. 2-3). The local community of bees in Tony Grove is more diverse than what was found interacting with ANBs. The most common bumble bee in the local community is *B. bifarius,* but we did not find this in any ANB. Overall the difference in bumble bee species detected in ANBs compared to the overall community is significantly different ($\chi^2 = 86.242$; df = 11; $p < 0.001$).



	Community	Species Interacting with ANBs
■ B. appositus	28	27
■ B. bifarius	104	0
■ B. centralis	22	5
■ B. fervidus	3	0
■ B. flavifrons	2	0
■ B. huntii	4	4
■ B. insularis	39	0
■ B. melanopygus	1	0
■ B. mixtus	5	3
■ B. occidentalis	3	0
■ B. rufocinctus	4	3
■ B. sylvicola	1	0
■ unknown Bombus	0	28

Figure 2-3. Stacked bar plot showing the abundance of bees found in nest boxes and from net surveys. The top bar represents bees found in nest boxes and the bottom bar represents the community of bees in the study site.

Psithyrus usurpation

Bombus (*Psithyrus*) *insularis* was the only socially parasitic species detected in ANBs, totaling seven usurpations detected over two years (Table 2-8). Most were found to parasitize *B. appositus* nests (14.8%), but a higher proportion of *B. insularis* occurred in *B. centralis* nests (40%).

Table 2-8

Table showing the number of boxes that had colonies that contained the social parasite *B. insularis.*

Presence of *B. insularis*	*B. appositus*	*B. centralis*	*B. huntii*	*B. mixtus*	*B. rufocinctus*	Total
Absent	23	3	3	3	3	35
Present	4	2	1	0	0	7

Other animals that utilized the boxes for nesting were chipmunks, Vespinae wasps, pacific wrens, ants and earwigs (Table 2-9). In 2016, Vespinae wasps nested in more boxes than any other animal taxa and 2017 had more chipmunks present in the nest boxes. The presence of Vespinae wasps did not exclude bumble bee nesting (5 boxes had both *Bombus* and wasp nests actively foraging simultaneously).

Table 2-9

Table showing all of the counts of animals that nested in ANBs. "Vespinae and *Bombus"* indicates ANBs that were cohabited by both wasp colonies and bumble bee colonies.

Animals in nest boxes	Count
Bombus	45
Vespinae	45
Vespinae and *Bombus*	5
Formicidae (ants)	4
chipmunk	15
Dermaptera (earwigs)	1
Pacific wren	12
none	77

Discussion:

A relatively high number of ANBs had bumble bees initiate nesting and a modest number of those went on to produce full colonies within the nest boxes. Of the 204 boxes deployed, 34% of them had detectable nest initiation activity from bumble bees and 16% of the nest boxes developed into colonies. *Bombus appositus* was the most common nesting species in the boxes (*B. appositus*; n = 27). Experiments testing the efficacy of constructed nesting sites have been done in Europe, New Zealand and in North America with varying results. Some studies show no nesting in nest boxes and some have shown over half of the man-made structures having had bumble bee queens interact with them (Johnson et al., 2019). This study focused on arboreal placement of nest boxes, but most studies have included them as a small subsample of nest boxes and have compared subterranean, surface level above-ground, and aerial or arboreal nest boxes. A total of six species of bumble bees used the boxes including one social parasite (*B. insularis*).

Neither blue/UV nest entrances on ANBs nor placement of nest boxes based on aspect increased nest initiation and/or colonies that reached social phase. Color vision in bees is shown to be strongly linked with foraging and associated with food rewards. This lack of nesting is evidence that color vision specifically the blue/UV reflectance in this study does not attract queens to nest boxes and is not an important cue for queen bumble bees searching for nesting sites. One can also infer that a blue/UV color entrance doesn't inhibit nesting in nest boxes either. Aspect of the nest box also showed no significant difference in both nest initiation events and ANBs that develop into social phase colonies. This is surprising since ANBs on south-facing slopes are where natural bumble bee nesting sites and foraging sites are likely to be available first due to this being the aspect with the earliest snow melt. Since nest boxes are arboreal this may negate the effect of earlier phenology on south-facing slopes. Finally, since ANBs have most of their surface area exposed to air then they are likely to encounter temperature changes on south facing slopes where there is less canopy density and more sun exposure, making nest boxes on the south slope more difficult to colonize and raise a colony within them. This may actually be because the species that interacted with ANBs were mostly *B. appositus*, a species with queens late to break diapause. The implications of *B. appositus*' breaking diapause later than other bumble bees is discussed later in this section. Regardless of the lack of significant results, this study had its highest number of bumble bee interactions in 2016 in ANBs deployed on south-facing slopes without color entrances. This high number of interactions by bumble bees with nest boxes shows that arboreal placement may be one of the better types of deployment for nest boxes in wildlands.

Bombus appositus was the predominant nesting species in ANBs with 66.7% of ANBs containing mature social phase *B. appositus* colonies. The species richness of bumble bees that would nest in ANBs did not reflect that of the surrounding community (Tony Grove) of bumble bees. *Bombus appositus* is an abundant bumble bee species, but the most common species in Tony Grove is *B. bifarius*. Hobbs 1966 notes that *B. appositus* in Alberta readily nested in both underground and surface level nests, and concludes that *B. appositus* is less specialized in its nest site selection. Also worth mentioning is that according to unpublished data utilizing three nest box types (underground, ground surface, and arboreal), *B. appositus* only nested in aboveground nest boxes. *B. appositus* may be more specialized for arboreal nesting in the Wasatch Range of Utah than in Alberta based on the high number of nest boxes inhabited by this species in the current study. Among this rich bumble bee community of Tony Grove are species that have experienced notable decline such as *B. occidentalis* (Cameron et al., 2011; Rhoades et al., 2016) however no species of concern were found nesting in the ANBs.

Bombus appositus readily nesting in ANBs may provide an opportunity to study a new study species of bumble bees for which little is known. Additionally, little is known of the subgenus *Subterranobombus* for which *B. appositus* is a part of. Augmentation of nesting in wild lands can be used to help manage endangered plants by providing supplemental pollination service in Tony Grove and other similar habitats. *Bombus appositus* is abundant in the high elevation areas of the intermountain west, Rocky Mountains, the Sierra Nevada, and high elevation subalpine and alpine environment (Koch et al 2012). This species is one of three representatives of the subgenus

Subterranobombus found in the US (the other species being *B. borealis* and *B. distinguendus,* a mostly Holarctic species that extends into the new world on Attu Island in the Aleutian Arc of Alaska (Williams et al., 2014). *Bombus appositus* is a pollinator of *Cirsium, Delphinium, Linaria, Trifolium, Geranium,* and *Penstemon* (Hobbs 1966; Koch et al 2012) therefore ANBs may increase fecundity through pollination of common and rare plants in these genera within similar subalpine plant communities. Hobbs (1966) and Plowright (1966) note that in Canada and in the United States, *B. appositus* is a woodland species. The current study area, Tony Grove, is a mosaic of alpine meadows and subalpine woodland with aspen groves and stands of conifers which provide an excellent habitat for this species. *Bombus appositus* spring queens are observed later in the season than other bumble bees occurring in the same habitat as them (Hobbs 1966; Koch et al., 2012).

Late nest-searching may be a reason this species may be taking to these boxes over the other species in the community is that there may be limited sites for nesting at the point that this bee breaks out of diapause. Also, by the time *B. appositus* queens are searching for nests, undergrowth can be in excess of 1 m tall, on the southern slopes of Tony Grove. The height of the vegetation may influence the search image for finding nesting sites in spring *B. appositus* queens and these ANBs are potentially elevated at a height that is optimal for the search image of the late vernal queens of *B. appositus*. Hobbs (1966) also notes that *B. appositus* produces small colonies which may not be optimal, in that this species will not produce many workers that will pollinate plants on the landscape. Overall, these results are exciting because they show that *B. appositus* prefers these ANBs substantially more than other species, which may provide unique opportunities to observe and understand the colony development of this species.

A large proportion of the ANBs showed signs of bumble bee interaction without positive identification of the species. These missed identifications should be clarified for future use in understanding more about which species interact with the ANBs for further optimization of this method. While observations of the nest boxes were conducted during the day, it would have been more effective to survey the nest boxes at night when bees would not be out foraging. This would also increase the incidents of early observations of bumble bees that enter nest boxes, but do not leave pollen provisions.

It is important to consider whether ANBs could have negative consequences for bumble bee populations, because they may benefit species that compete for nesting sites with bumble bees. *Bombus insularis* is a common bumble bee species within the subgenus *Psithyrus*, a subgenus of bumble bee where all species are adapted to being social parasites. Social parasitism within *Psithyrus* occurs when a female *Psithyrus* species invades established nests from another species of bumble bee (Lhomme & Hines, 2018, 2019). The *Psithyrus* female then usurps the colony by killing the foundress queen, often before the colony develops enough for the host foundress queen to produce reproductive castes (Lhomme & Hines, 2018, 2019). The usurping female *Psithyrus* do not lay non-reproductive (worker) castes and do not collect pollen for the colony they usurp skipping the social phase and the colony development phases of a typical bumble bee life cycle (Goulson 2010; Lhomme et al. 2013). Alford (1975) noted that nest boxes placed off of the ground may expose more colonies than usual to *Psithyrus* usurpation because of how exposed they are to nest-searching *Psithyrus* females. This experiment showed that 20% of colonies were exposed and usurped by the generalist social parasite *B. insularis.* An interesting finding is that *B. insularis* were found in 2.8% of nests that had not reached the social phase of development. This illustrates the importance of phenology between bumble bee host and the bumble bee social parasites, because *Psithyrus* rely on colonies that mature to the social phase so that the foundress' workers will care for the *Psithyrus*' offspring. Even though usurpation by *Psithyrus* may be an impediment to increasing non-parasitic bumble bees, it could also provide an opportunity to understand more about socially parasitic bumble bees (Lhomme et al., 2013; Martin et al., 2010). Removing ANBs containing *Psithyrus* usurped colonies can mitigate the effects of increasing social parasite abundance and simultaneously provide opportunities to observe social parasitism within bumble bees of which very little is known (Lhomme et al. 2013; Lhomme and Hines 2019).

Both interspecific and intraspecific usurpation of nests may be high due to increased exposure of ANBs and due to facultative social parasitism in non-*Psithyrus* species (Fisher 1987). Intraspecific usurpation or attempted usurpation was assumed to have taken place often in *B. appositus* nests due to at least one dead *B. appositus* queen found in all social phase *B. appositus* nests. *Bombus appositus* may carry out intraspecific usurpation naturally, possibly because this species has later vernal queens (Fisher 1987). However, it is possible the frequency of nest usurpation increases in ANBs. Further investigation of ANBs needs to be conducted to model whether ANBs can be used for conservation efforts in supporting bumble bee populations, particularly in the face of possible increased exposure to social parasites and usurping congeneric bumble bees.

Many other organisms besides bumble bees were detected in ANBs. These include wasps in the subfamily Vespinae, Chipmunks, and Pacific wrens. Making the nest entrance smaller may be an effective way to exclude these organisms from nesting since they do not cohabitate nest boxes. No nest boxes with any vertebrate present had any bumble bees nesting in the box after the vertebrates started nesting. An interesting aside for the vertebrate nesters is that the only bird species that was known to nest in these boxes were Pacific Wrens (*Troglodytes pacificus*). Although some nesting of birds in these boxes was expected, it was surprising that the pacific wren was the only species that could nest in the box. The small entrance is likely a cause of the pacific wren nesting fidelity in ANBs. This population of pacific wren is an interesting population because it is a small isolated year round resident population (Sibley 1961). Deploying these boxes for these birds is an opportunity to observe this isolated population and understand differences that may occur in nesting in this population compared to the Pacific Northwest population. For land managers seeking to increase these insectivorous birds in the Wasatch Range, this ANB design may be improved upon to increase nesting of these birds instead of bumble bees. The previous 2009 sampling showed that ants nested in 8.4% of partially buried boxes in Tony Grove. Birds such as chickadees (*Parus*) will nest in arboreal boxes meant for birds, showing that there is competition amongst birds and bumble bees for nest sites (Bowles 1909). It was mentioned earlier that often bumble bees utilize old rodent nests for nesting sites, however these old rodent nests are also ideal for rodents to reuse or takeover and rodents will often compete for nesting sites with bumble bees or even destroy bumble bee colonies (Hobbs et al. 1960). These vertebrate and invertebrate competitors alike need to be documented to understand competition for nest sites and the efficacy in attracting bumble bees versus other animals that compete with bumble bees.

Vespinae wasps were a prominent species within nest boxes, especially in 2016. Vespinae species are likely undesirable inhabitants for anyone managing for wild bumble bee species. Simple design modifications of ANBs can help reduce the number of nesting wasps, because Vespinae wasps build nests from the top down. Lining the ceiling of the nest box with either a removable layer of material or material that make nest building difficult for wasps if boxes are deployed in locations that cannot be regularly checked. One would assume that Vespinae wasps would inhibit simultaneous nesting with bumble bees, but this experiment showed that bumble bees and wasps shared ANBs on occasion (n = 5). This has also been observed in eastern United States bumble bee communities where similar ANBs have been deployed for bumble bees (Slatkosky et al, personal communication). The only species that cohabitated with wasps in this study were *B. appositus*, but this may just reflect the frequency with which this species used ANBs in general. However, seeing as *B. appositus* may also be an arboreal nester, it is possible these interactions may occur naturally as well. One of the cohabitation nests produced relatively large colonies of both the Vespinae wasps and *B. appositus*. This relationship needs further study to understand how often bumble bees and wasps cohabitate nesting sites in arboreal cavities.

Overall, providing these ANBs for nesting bumble bees shows promise for bees in the montane subalpine environments of the Wasatch Range, specifically for recruiting nesting *B. appositus*. This approach should be repeated in other similar nearby environments to investigate whether this is a site specific phenomena or an effective means to bolster nesting habitat for a variety of bumble bee species.

Conclusion:

This experiment demonstrated that vernal bumble bee queens in the Cache-Wasatch subalpine environment are attracted to and can successfully nest in arboreal nest boxes (ANBs). I did not find any evidence that landscape properties such as slope aspect or visual stimuli such as blue/UV color entrances influence how many bumble bee queens initiate nests or how many bumble bee queens are able to develop mature colonies. *Bombus appositus* interacted with more ANBs than any of the five species that initiated nesting (non-*Psithyrus*) and had more nests enter into social phase, suggesting this species is particularly adept at utilizing ANBs. This experiment shows that ANBs are effective in attracting bumble bee queens, specifically *B. appositus* and can serve as a tool for creating habitat for this species of bumble bee. Many nest box studies explore the utility of nest boxes placed on the ground, but this study suggest future efforts should focus on ANBs across different habitats to test their efficacy in providing optimal nesting habitat for these important pollinators.

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Literature cited:

Ackerman, J. D. (1981). Pollination Biology of Calypso bulbosa var. occidentalis (Orchidacae): A food-deception system. *Madrona*, *28*(3), 101–110. Retrieved from http://www.jstor.org/stable/41424311

Alford, D. V. (1975). *Bumblebees*. London: Davis-Poynter.

Baranauskas, K. (2009). The Use of Bat Boxes of Two Models by Nathusius' Pipistrelle (Pipistrellus nathusii) in Southeastern Lithuania. *Acta Zoologica Lituanica*, *19*(1), 3–9. https://doi.org/10.2478/v10043-009-0002-y

Barron, M. C., Wratten, S. D., & Dovovan, B. J. (2000). A four-year investigation into the effiicacy of domiciles for enhancement of bumble bee populations. *Agricultural and Forest Entomology*, *2*, 141–146.

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1). https://doi.org/10.18637/jss.v067.i01

Bender, J., Fidino, M., Limbrick, K., & Magle, S. (2016). Assessing Nest Success of Black-Capped Chickadees (Poecile atricapillus) in an Urban Landscape Using Artificial Cavities. *The Wilson Journal of Ornithology*, *128*(2), 425–429. https://doi.org/10.1676/1559-4491-128.2.425

Billings, W. D., & Bliss, L. C. (1959). An Alpine Snowbank Environment and Its Effects on Vegetation, Plant Development, and Productivity. *Ecology*, *40*(3), 388–397. https://doi.org/10.2307/1929755

Bortolotti, G. R. (1994). Effect of nest-box size on nest-site preference and reproduction in American kestrels. *Journal of Raptor Research*, *28*(3), 127–133. Retrieved from https://www.google.com/search?q=issn%0892-1016

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., & Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, *108*(2), 662–667. https://doi.org/10.1073/pnas.1014743108

Crowther, L. P., Hein, P.-L., & Bourke, A. F. G. (2014). Habitat and Forage Associations of a Naturally Colonising Insect Pollinator, the Tree Bumblebee Bombus hypnorum. *PLOS ONE*, *9*(9), 1–10. https://doi.org/10.1371/journal.pone.0107568

Cusser, S., & Goodell, K. (2013). Diversity and distribution of floral resources influence the restoration of plant-pollinator networks on a reclaimed strip mine. *Restoration Ecology*, *21*(6), 713–721. https://doi.org/10.1111/rec.12003

Donovan, B. J., & Wier, S. S. (1978). Development of hives for field population increase, and studies on the life cycles of the four species of introduced bumble bees in New Zealand. *New Zealand Journal of Agricultural Research*, *21*, 733–756. https://doi.org/10.1080/00288233.1978.10427476

Fye, R. E., & Medler, J. T. (1954). Field domiciles for bumblebees. *Journal of Economic Entomology*, *47*(4), 672–676. https://doi.org/10.1093/jee/47.4.672

Gillott, C. (2003). Male Accessory Gland Secretions: Modulators of Female Reproductive Physiology and Behavior. *Annual Review of Entomology*, *48*(1), 163–184. https://doi.org/10.1146/annurev.ento.48.091801.112657

Goulson, D. (2003). Effects of Introduced Bees on Native Ecosystems. *Annual Review of Ecology, Evolution, and Systematics*, Vol. 34, pp. 1–26. https://doi.org/10.1146/annurev.ecolsys.34.011802.132355

Goulson, D. 2010. *Bumblebees: Behaviour, ecology, and conservation*. Oxford: Oxford University Press

Hatfield, R. G., & Lebuhn, G. (2007). Patch and landscape factors shape community assemblage of bumble bees, *Bombus* spp . (Hymenoptera : Apidae), in montane meadows. *Biological Conservation*, *139*, 150–158. https://doi.org/10.1016/j.biocon.2007.06.019

Heinrich, B. (1979). *Bumblebee economics*. Cambridge: Harvard University Press

Heinrich, B. (1984). Learning in Invertebrates. In P. Marler & H. S. Terrace (Eds.), *The Biology of Learning* (pp. 135–147). Berlin, Heidelberg: Springer Berlin Heidelberg.

Hobbs, G. A. (1966). Ecology of Species of Bombus Latr. (Hymenoptera: Apidae) in Southern Alberta. *The Canadian Entomologist*, *98*(3), 288–294.

Hobbs, G. A., Virostek, J. F., & Nummi, W. O. (1960). Establishment of Bombus spp. (Hymenoptera: Apidae) in Artificial Domiciles in Southern Alberta. *The Canadian Entomologist*, *92*(11), 868–872. https://doi.org/10.4039/Ent92868-11

Honkavaara, J., Koivula, M., Korpimäki, E., Siitari, H., & Viitala, J. (2002). Ultraviolet vision and foraging in terrestrial vertebrates. *Oikos*, *98*(3), 505–511. https://doi.org/10.1034/j.1600-0706.2002.980315.x

Jacobson, M. M., Tucker, E. M., Mathiasson, M. E., & Rehan, S. M. (2018). Decline of bumble bees in northeastern North America, with special focus on Bombus terricola. *Biological Conservation*, *217*, 437–445. https://doi.org/10.1016/j.biocon.2017.11.026

Johnson, S. A., Tompkins, M. M., Tompkins, H., & Colla, S. R. (2019). Artificial Domicile Use by Bumble Bees (Bombus; Hymenoptera: Apidae) in Ontario, Canada. *Journal of Insect Science*, *19*(7), 1–5. https://doi.org/10.1093/jisesa/iey139

Jones, J. R., Kaufmann, M. R., & Richardson, E. A. (1985). Effects of water and temperature. In *Aspen: Ecology and Management in the Western United States* (pp. 71–76).

Koch, J., J. Strange, and P. Williams. 2012. Bumble bees of the western United States. Publication No. FS-972. USDA Forest Service Research Notes, Fort Collins, Colorado, USA.

Lhomme, P., & Hines, H. M. (2018). Reproductive Dominance Strategies in Insect Social Parasites. *Journal of Chemical Ecology*, *44*(9), 838–850. https://doi.org/10.1007/s10886-018-0971-z

Lhomme, P., & Hines, H. M. (2019). Ecology and evolution of cuckoo bumble bees. *Annals of the Entomological Society of America*, *112*(3), 122–140. https://doi.org/10.1093/aesa/say031

Lhomme, P., Sramkova, A., Kreuter, K., Lecocq, T., Rasmont, P., & Ayasse, M. (2013). A method for year-round rearing of cuckoo bumblebees (Hymenoptera: Apoidea: Bombus subgenus Psithyrus). *Annales de La Société Entomologique de France*, *49*(1), 117–125. https://doi.org/10.1080/00379271.2013.774949

Lye, G. C., Park, K. J., Holland, J. M., & Goulson, D. (2011). Assessing the efficacy of artificial domiciles for bumblebees. *Journal for Nature Conservation*, *19*(3), 154–160. https://doi.org/10.1016/j.jnc.2010.11.001

Malfi, R. L., Crone, E., & Williams, N. (2019). Demographic benefits of early season resources for bumble bee (B. vosnesenskii) colonies. *Oecologia*, *191*(2), 377–388. https://doi.org/10.1007/s00442-019-04472-3

Martin, S. J., Carruthers, J. M., Williams, P. H., & Drijfhout, F. P. (2010). Host Specific Social Parasites (Psithyrus) Indicate Chemical Recognition System in Bumblebees. *Journal of Chemical Ecology*, *36*(8), 855–863. https://doi.org/10.1007/s10886-010-9805-3

Martinet, B., Lecocq, T., Brasero, N., Gerard, M., Urbanová, K., Valterová, I., … Rasmont, P. (2019). Integrative taxonomy of an arctic bumblebee species complex highlights a new cryptic species (Apidae: Bombus). *Zoological Journal of the Linnean Society*. https://doi.org/10.1093/zoolinnean/zlz041

Ostevik, K. L., Manson, J. S., & Thomson, J. D. (2010). Pollination Potential of Male Bumble Bees (Bombus impatiens): Movement Patterns and Pollen - Transfer Efficiency. *Journal of Pollination Ecology*, *2*(4), 21–26. Retrieved from http://dx.doi.org/10.26786/1920-7603%282010%293

Pitts-Singer, T. L., Hanula, J. L., & Walker, J. L. (2002). Insect Pollinators of Three Rare Plants in a Florida Longleaf Pine Forest. *Florida Entomologist*, *85*(2), 308–316. https://doi.org/10.1653/0015-4040(2002)085[0308:IPOTRP]2.0.CO;2

Plowright R.C & Jay, S.C. (1966) Rearing Bumble Bee Colonies in Captivity, Journal of Apicultural Research, 5:3, 155-165, DOI: 10.1080/00218839.1966.11100149

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Pywell, R. F. (2005). Providing foraging resources for bumblebees in intensively farmed landscapes. *Biological Conservation*, *121*, 479–494. https://doi.org/10.1016/j.biocon.2004.05.020

Radunzel, L. A., Muschitz, D. M., Bauldry, V. M., & Arcese, P. (1997). A Long-Term

Study of the Breeding Success of Eastern Bluebirds by Year and Cavity Type (Estudio de Largo Alcance Sobre el Éxito Reproductivo de Sialia sialis en Distintos Tipos de Cajas de Anidamiento). *Journal of Field Ornithology*, *68*(1), 7–18. Retrieved from https://www.jstor.org/stable/4514186

Raine, N. E., & Chittka, L. (2005). Colour Preferences in Relation to the Foraging Performance and Fitness of the Bumblebee Bombus terrestris. *Uludag Bee Journal*, *5*(4), 145–150.

Rhoades, P. R., Koch, J. B., Waits, L. P., Strange, J. P., & Eigenbrode, S. D. (2016). Evidence for Bombus occidentalis (Hymenoptera: Apidae) populations in the olympic peninsula, the palouse prairie, and forests of Northern Idaho. *Journal of Insect Science*, *16*(1), 1–5. https://doi.org/10.1093/jisesa/iev155

Richards, K. W. (1978). Nest site selection by bumble bees (Hymenoptera: Apidae) in southern Alberta. *The Canadian Entomologist*, *110*(3), 301–318. https://doi.org/10.4039/Ent110301-3

Sibley, David, 1961-. *The Sibley Guide to Birds*. New York: Alfred A. Knopf, 2000

Sircom, J., Arul Jothi, G., & Pinksen, J. (2018). Monitoring bee populations: are eusocial bees attracted to different colours of pan trap than other bees? *Journal of Insect Conservation*. https://doi.org/10.1007/s10841-018-0071-y

Sladen, F. 1912. *The Humble-Bee: Its Life-History and How to Domesticate it, with Descriptions of All the British Species of Bombus and Psithyrus* (Cambridge Library Collection - Zoology). Cambridge: Cambridge University Press. doi:10.1017/CBO9781107705739

Spivak, M., Mader, E., Vaughan, M., & Euliss, N. H. (2011). The plight of the bees. *Environmental Science and Technology*, *45*(1), 34–38. https://doi.org/10.1021/es101468w

Strange, J. P., & Tripodi, A. D. (2019). Characterizing bumble bee (Bombus) communities in the United States and assessing a conservation monitoring method. *Ecology and Evolution*, *9*(3), 1061–1069. https://doi.org/10.1002/ece3.4783

Tuomi, J., Lämsä, J., Wannas, L., Abeli, T., & Jäkäläniemi, A. (2015). Pollinator behaviour on a food-deceptive orchid Calypso bulbosa and coflowering species. *Scientific World Journal*, *2015*, 1–9. https://doi.org/10.1155/2015/482161

Valterová, I., Martinet, B., Michez, D., Rasmont, P., & Brasero, N. (2019). Sexual attraction: a review of bumblebee male pheromones. *Zeitschrift Für Naturforschung C*, *74*(9–10), 233–250. https://doi.org/10.1515/znc-2019-0003

Velthuis, H. H. W. W., & van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, *37*(4), 421–451. https://doi.org/10.1051/apido:2006019

Viitala, J., Korplmäki, E., Palokangas, P., & Koivula, M. (1995). Attraction of kestrels to vole scent marks visible in ultraviolet light. *Nature*, *373*(6513), 425–427.

https://doi.org/10.1038/373425a0

Vogt, F. D., Heinrich, B., Dabolt, T. O., & McBath, H. L. (1994). Ovary development and colony founding in subarctic and temperature-zone bumblebee queens. *Canadian Journal of Zoology*, *72*(9), 1551–1556. https://doi.org/10.1139/z94-206

Vogt, F. D., Heinrich, B., & Plowright, C. (1998). Ovary development in bumble bee queens: The influence of abdominal temperature and food availability. *Canadian Journal of Zoology*, *76*(11), 2026–2030. https://doi.org/10.1139/z98-146

Williams, P. H., & Osborne, J. L. (2009). Bumblebee vulnerability and conservation world-wide. *Apidologie*, *40*(3), 367–387. https://doi.org/10.1051/apido/2009025

Williams, P. H., Thorp, R. W., Richardson, L. L., & Colla, S. R. (2014). *Bumble Bees of North America*. Retrieved from http://www.jstor.org/stable/j.ctt6wpzr9

CHAPTER 3

REPRODUCTIVE DEVELOPMENT OF MALE *BOMBUS VOSNESENSKII* RADOZKOWSKI

Abstract:

Bumble bees, genus *Bombus* (Latrielle), are important pollinators for agricultural and wild flowering plants. Several species of bumble bees are produced and moved worldwide for commercial pollination of several crops. Understanding *Bombus* reproductive biology is a critical aspect of commercial breeding, but our knowledge of this is restricted primarily to females from limited species. I investigated morphological changes of the male internal reproductive apparatus (MIRA) in a species of commercial interest, *Bombus vosnesenskii*, for which little reproductive biology is known. Age-related changes in three of the major structures of the MIRA, the testes, the accessory testes, and the accessory glands, were studied in male bees that ranged from 0-14 days post-eclosion. Testes size diminished rapidly from eclosion until bees were eight days old, and this corresponded with a transfer of sperm to the accessory testes where sperm was present in all bees sampled at eight days old. The size of the accessory gland remained stable through reproductive maturation but varied with the size of the male bee. Sperm viability assays showed a large amount of sperm being produced in *B. vosnesenskii* without much variation of sperm present between age groups. Based on the timing of these observations, I conclude that male *B. vosnesenskii* are not fully sexually mature before they are eight days old. These patterns of reproductive maturation were similar for males produced by workers in microcolonies and those produced by queen-right colonies, indicating that timing of mating does not need to be adjusted when breeding males are produced by workers. This is the first study to document changes of the MIRA of bumble bees, provides a baseline for future studies of reproduction in male bumble bees, and guidance when selecting males for captive breeding.

Keywords: bumble bees, reproduction, reproductive development, testes, accessory testes, accessory glands

Introduction:

Modern captive rearing of bumble bee colonies began early in the 20th century with Sladen (1912). However, the first commercial line of bumble bees began with the European species *Bombus terrestris* in 1987 (Velthuis & van Doorn 2006). Since then, bumble bee colony production has become a multi-million-dollar industry and an important contribution to food security in pollination of crops in controlled environment (e.g., greenhouse agriculture) and open field agriculture (Banda & Paxton, 1991; Strange, 2015; Velthuis & van Doorn, 2006). Captive colonies used for pollination in agricultural environments primarily consist of two species of bumble bees, *B. impatiens* and *B. terrestris*, which both pollinate a wide variety of crops (Artz & Nault, 2011; Banda & Paxton, 1991; Desjardins & De Oliveira, 2009; Morandin et al., 2001; Sapir et al., 2019; Strange, 2015; Stubbs & Drummond, 2001; Sutherland et al., 2017; Velthuis & van Doorn, 2006; Vergara & Fonseca-Buendía, 2012; Whittington et al., 2004; Zhang et al., 2015). These commercial colonies provide pollination outside of the natural growing season by pollinating plants grown in greenhouses. Greenhouse pollination continues to increase in production and over half of the crops grown in green houses worldwide require buzz pollination from bumble bees (Lensing, 2018; Thornsbury et al. 2016). Thus, the demand for commercial rearing of bumble bees to accommodate pollination needs continues to increase.

Challenges caused by expanding captive breeding of bumble bees include potential threats to native bumble bee communities (Byatt et al., 2016; Ings et al., 2006) from both the direct competition of non-native bees in the environments (Morales et al. 2013; Looney et al. 2019) and the spread of pathogens (Cameron et al., 2016; Goka et al., 2001; Graystock et al., 2013). Breeding facilities have the potential to release bees into the surrounding area (Byatt et al., 2016; Graystock et al., 2013; Otterstatter & Thomson, 2008; Whittington et al., 2004). If the species being bred is non-native, direct competition with native species is expected (Aizen et al., 2018; Kanbe et al., 2008; Morales et al., 2013). Both of the commercially available *Bombus* species have now established populations outside of their native range (Looney et al., 2019; Morales et al., 2013; Stout & Morales, 2009). *Bombus terrestris* has caused declines in native bumble bee populations in South America (Morales et al. 2013), and hybridized with native species in Japan (Kanabe et al., 2008). *Bombus terrestris* have established in areas where no native bumble bee fauna existed previously, such as New Zealand and Tasmania, where they pollinate and propagate non-native plants (Buttermore et al. 2015; Donovan and Wier 1978; Stout & Goulson 2000).

Cultivated colonies may also pose a health risk to local species if they harbor pathogens or parasites to which native species have not been previously exposed (Cameron et al., 2011; Goka et al., 2001; Graystock et al., 2013; Murray et al., 2013;

Sachman-Ruiz & Reynaud, 2015; Velthuis & van Doorn, 2006). Phoretic mites and the queen castrating nematode *Sphaerularia bombi* have both been introduced as bumble bee species have been moved to novel environments (Goka et al., 2001; Meeus et al., 2011).

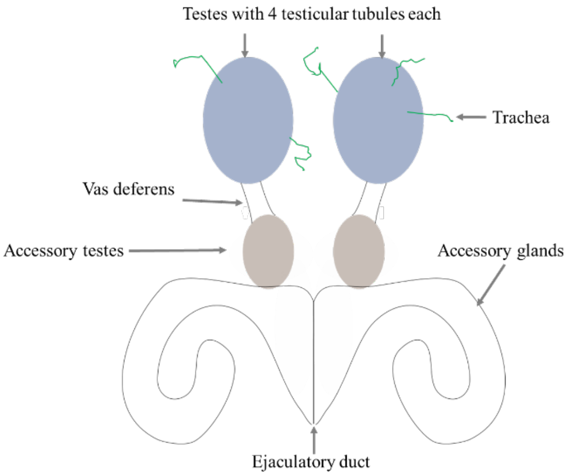
One way to mitigate the harmful effects of commercial propagation of bumble bees on local bee communities is to focus commercial efforts on species native to a given region. This requires the development of captive breeding methods for additional species, because *B. impatiens* is native to only eastern North America and *B. terrestris* is only native to Europe and the northern Africa. However, these species are currently being used for pollination services worldwide (Velthuis & van Doorn, 2006). Early attempts at domesticating a western North American species focused on the production of *B. occidentalis*, but this was unsuccessful due to the difficulty of raising colonies in captivity (Velthuis & van Doorn, 2006; Rao and Stephen 2007). Since *B. occidentalis* was unable to be sustained in captivity, the primary species used in North and Central America has been *B. impatiens* (Velthuis & van Doorn, 2006). Growing concerns of the ecological consequences of the use of this species have precipitated the development of other western North American bumble bees for commercial use (Ratti and Colla; Colla et al. 2008; Looney et al., 2019).

One western North American bumble bee species that has shown potential for commercial breeding is the Vosnesensky bumble bee (*B. vosnesenskii*) because of its extended native range (Jackson et al., 2018; Williams et al., 2014), large colony size (Malfi et al., 2019; Strange, 2015), and effectiveness at pollinating greenhouse crops such as tomatoes (Dogterom et al., 1998; Strange 2015). *Bombus vosnesenskii* occurs along the North American Pacific coast from far southern British Columbia to the northernmost part of the Baja peninsula in Mexico, and it does not occur much further east than the Sierra Nevada – Cascade mountain crest (Koch et al 2012). Vosnesensky bumble bee colonies have hundreds of workers, which makes them cost-effective for pollination (Malfi et al., 2019; Strange, 2015). Expanding efforts to commercialize the species would benefit from an understanding of the mating biology of the species, including both female and male sexual maturation.

Despite its potential as a managed pollinator species, continual captive rearing of *B. vosnesenskii* colonies has been unsuccessful for commercial producers of bumble bee colonies and researchers alike. This is largely because of the difficulty to get *B. vosnesenskii* to produce queens once colonies mature (personal observation). Even in commercialized species like *B. terrestris*, not all gynes go on to produce colonies. It is therefore important to select the highest quality males for mating with the available gynes (Velthuis & van Doorn, 2006). Like most other social Hymenoptera, mating is a brief period of the colony cycle. Yet this event has a huge impact on colony establishment and reproductive potential (Duvoisin et al., 1999; Velthuis & van Doorn, 2006).

While the reproductive physiology of female bumble bees has received some attention (Alaux et al., 2007; Duchateau & Velthuis, 1989; Vogt et al., 1994; Vogt et al., 1998), we know comparatively little about the timing of male reproductive maturation at any stage of the male bee’s lifecycle. Prior research of bumble bee reproductive biology is primarily focused on one species, *B. terrestris* of which the following information is based (Greeff & Schmid-Hempel, 2008; Tasei et al. 1998). Most bumble bee queens and many other social Hymenoptera are monandrous and the females copulate with males for a brief period during maturation (Baer et al., 2003). Females store viable sperm from their single mate to fertilize eggs over their one-year lifespan (Baer et al., 2003). A thorough understanding of male reproductive biology will result in higher success rates of captive breeding. The goal of this study is to describe temporal patterns of reproductive maturity in male *B. vosnesenskii* by quantifying changes in the male internal reproductive apparatus (MIRA) of *B. vosnesenskii*. Because little is known about this development in male bumble bees, this study simultaneously answers questions about specific developmental trends in *B. vosnesenskii* and the broader trends in MIRA development/diminishment occurring across all bumble bee males.

A)



B)

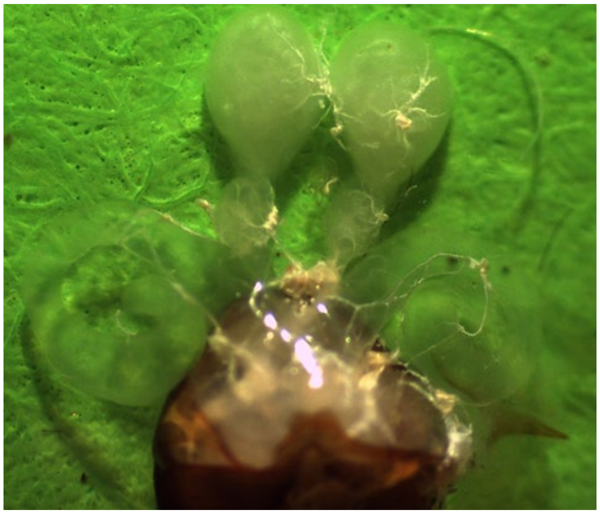


Figure 3-1. Ventral view of the bumble bee male internal reproductive apparatus (MIRA) with the anterior at the top and posterior portion at the bottom. A) Diagram showing the basic structures composing the MIRA. B) MIRA of *B. vosnesenskii* removed from the abdomen. This specimen was 1-day post-eclosion.

The bumble bee MIRA is composed of two sets of testes, accessory testes and accessory glands (Fig. 3-1) (Duchateau & Mariën, 1995; Duvoisin et al., 1999; Ferreira et al., 2004; Tasei et al., 1998). Spermatogenesis begins during the pupal stage and continues post-eclosion. Each of the two testes is an assemblage of four testis tubules encased within the scrotal membrane. These germ cells initiate spermatogenesis by undergoing multiple mitotic divisions to form mature sperm known as spermatozoa (Cruz-Landim ey al., 1980). After eclosion, the spermatozoa migrate from the testes to the accessory testes, where they are stored until ejaculation. Hymenopteran insects produce a finite number of spermatozoa as germ cells terminate during spermatogenesis (Cruz-Landim et al., 1980). As a result, the testes diminish once all mature spermatozoa have migrated to the accessory testes, which takes place post-eclosion. The age-based patterns of this diminishment has not been quantified in any bee species (Duchateau & Mariën, 1995; Duvoisin et al., 1999; Ferreira et al., 2004; Snodgrass, 1910). This diminishment is linked with reproductive maturation and can provide evidence for when male bumble bees are reproductively mature along with documentation of when sperm is present in the accessory testes.

Another important component of the bumble bee ejaculate is the seminal fluid, which is primarily produced in the accessory glands. During mating, mature sperm and seminal fluid are transferred to females to facilitate sperm entering the spermatheca, a specialized organ where the gyne stores sperm for starting a colony the following spring. Seminal fluid from the accessory glands has many functions in social hymenopterans beyond creating a medium for sperm motility. It also functions in altering the behavior and physiological processes of the female post-copulation (Liberti et al., 2019; Mikheyev, 2004). The seminal fluid of *B. terrestris* delivers a lipid based “mating plug” that reinforces monandry of the females by preventing secondary copulations from successful sperm transfer to the female’s spermatheca (Baer et al., 2001; Duvoisin et al., 1999; Korner 2003). Although accessory glands appear to be fully developed post-eclosion, it is unknown how the size of this gland changes with age or body size. In this study, I measured accessory gland length to detect any changes that may occur as adult males become reproductively mature.

While knowing the age that accessory testes fill with spermatozoa and become opaque indicates development in adults, knowing when the maximum number of viable spermatozoa are in the accessory testes is a more accurate measure of peak mating potential. Quantifying how many viable spermatozoa are present provides a more detailed assessment of when males are of their highest mating quality, as early in development sperm may not be mature and later in development sperm cells may die. I measured the viable sperm count within the accessory testes of males to know at what age males had the highest viable spermatozoa counts.

An additional limitation to mating success in captivity is timing the availability of males to that of when mature colonies are producing females. A commonly used technique is thus to produce males from “microcolonies” (Regali, et al., 1995; Klinger et al. 2019). Microcolonies are queen-less colonies composed of several workers where one worker exerts social dominance and begins laying unfertilized eggs, while the other workers remain sterile and care for the resulting male brood (Klinger et al. 2019). Queen bumble bees have much larger amounts of fat body and ovariole investment than workers do (Alford 1975) and thus, worker-laid males are typically rare in wild bumble bee colonies (Huth-Schwarz et al., 2011), though workers can sometimes lay their own unfertilized eggs toward the end of the colony life cycle (Cnaani et al., 2002). Given the large differences in physiology between queens and workers, it is possible that males that develop from worker- and queen-laid eggs could have important phenotypic differences. A successful captive rearing program requires knowledge of any differences in the timing of male reproductive maturity that may stem from whether they were laid by queens or workers. I used males from microcolonies and males from queen-right colonies to investigate differences in MIRA between males of these colony types.

I investigated the timing of reproductive maturation in male *B. vosnesenskii* by quantifying morphological changes in the MIRA and sperm maturation as a function of age. Specifically, I measured the surface area of the testes and accessory testes in relationship to one another as a metric of sperm maturation and migration. An additional metric of maturation is the observation of which age groups had opaque accessory testes indicating stored mature sperm inside of them. A final observation of the testes included whether or not the scrotal membrane was predominately covered by trachea. This was an additional feature noticed in older males and I sought to document when it occurs in what age-group of males. I also documented changes in the length of the accessory gland to determine whether it undergoes changes in size to prepare for mating. Sperm viability was measured on some age groups of male *B. vosnesenskii* from queen-right colonies to investigate when the highest sperm counts are present in *B. vosnesenskii*.

Methods:

Producing males of known ages post-eclosion

To produce males of known ages, whole colonies of bumble bees needed to be produced in a lab setting to closely monitor which day eclosion occurred for each male within the colony they were produced in. Males were produced using two methods of production to look for differences in development between worker-laid males and males from a standard queen-right colony. To ensure males were laid by workers, microcolonies were produced and males were pulled directly from queen-right colonies. Both methods of production require raising natal colonies from solitary queens but detail in this part of male production can only be provided for microcolony males because all queen-right males used were delivered from Biobest Inc. which uses a patented methods for raising bumble bee colonies from solitary queens. Therefore, details on bumble bee rearing only apply to males produced from microcolonies. In total males, came from 10 natal colonies with worker-laid males coming from six of those natal colonies and queen-right produced males coming directly from four natal colonies.

Worker-laid male production from microcolonies

Queen *B. vosnesenskii* were collected while foraging or flying using aerial insect nets, transferred to shipping vials and placed in a cooler for transport to the USDA-ARS Pollinating Insect Research Unit (PIRU) bumble bee rearing facility. At PIRU, queens were then induced to produce colonies following Evans, Burns, and Spivak (2007) with the following modifications. Each queen was given a unique identification code and placed in 2.25 L plastic queen initiation boxes (Biobest Canada Ltd., Leamington, ON) with approximately 500 mg of honey bee collected pollen and sugar syrup *ad libitum*.

Sugar was fed in a 60 mL plastic reservoir as a 50% total sugar solution containing sucrose:fructose:glucose at 2:1:1 and containing 0.5% by volume of sorbic acid to prevent spoilage, 0.5% Honey B Healthy® (Honey-B-Healthy, Inc. Cumberland, MD) as a feeding stimulant, and 0.5% Amino-B Boost® (Honey-B-Healthy, Inc. Cumberland, MD) as a supplement of amino acids. Initiation boxes were maintained in the dark at 28°C ± 1° and at 55-60% humidity (Strange 2010). Queens were checked daily for signs of nesting behavior, including wax secretion, honey pot construction, or presence of brood and workers. Once five adult workers had eclosed, the colony was moved into a 7.75 L plastic hive box (Biobest Canada Ltd., Leamington, ON).

When queen-right colonies contained 40-50 adult workers, microcolonies were created following the same protocol of Klinger et al (2019). Five workers were removed from the natal nest and placed in 2.25 L plastic queen initiation boxes and given pollen to stimulate oviposition (Regali & Rasmont, 1995). Microcolonies were checked daily for eclosed males, which we removed from the microcolony and placed in another container labeled with their parental colony ID and the date that they were collected. These males were provided pollen and lab-made sugar water solution (described above) *ad libitum* to sustain them as they aged. Age groups of male bees ranged from callow (i.e., newly eclosed) up to 14 days post-eclosion. These males are referred to as microcolony males.

Queen-right colonies

Additional male *B. vosnesenskii* from queen-right colonies were provided by Biobest USA, Inc. 1-day post-eclosion. Ten bees were dissected the day of arrival for an age grouping of 1-day old bees. The rest of the male bees were stored in empty queen-initiation boxes mentioned in the previous paragraph and kept in the dark at 28°C ± 1°

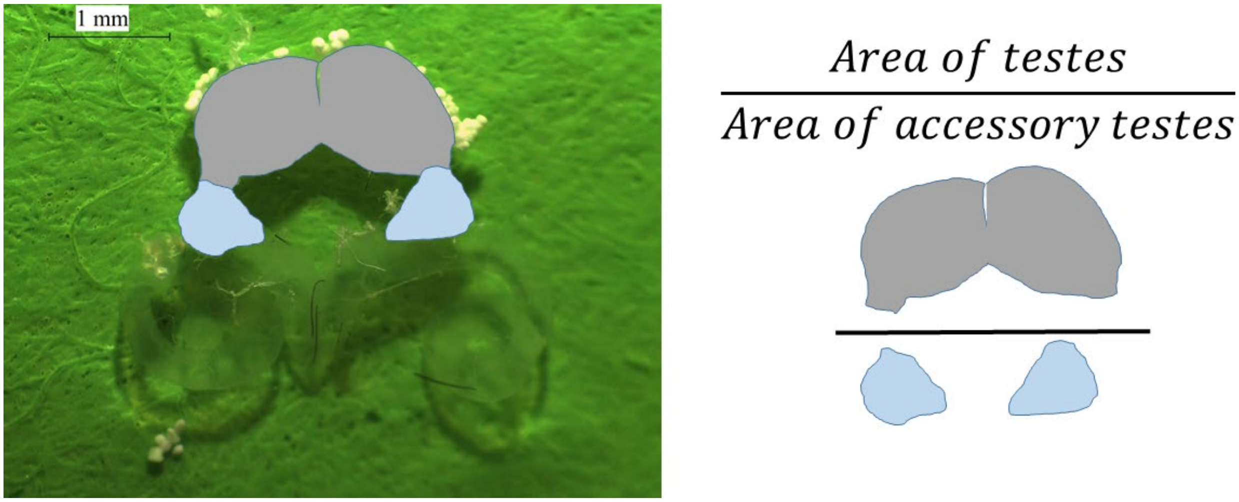
and at 55-60% humidity. These remaining males were stored until becoming 4, 7, or 10 days old post-eclosion. Once bees reached these one of these ages they were dissected, providing a 4 ages groups of all queen-right males sampled. Ten bees were dissected from each of the age groups for a total of 40 males dissected from Biobest queen-right colonies. All males in this study were virgin males and were never exposed to gynes once removed from their queen-right colony.

Dissection procedures

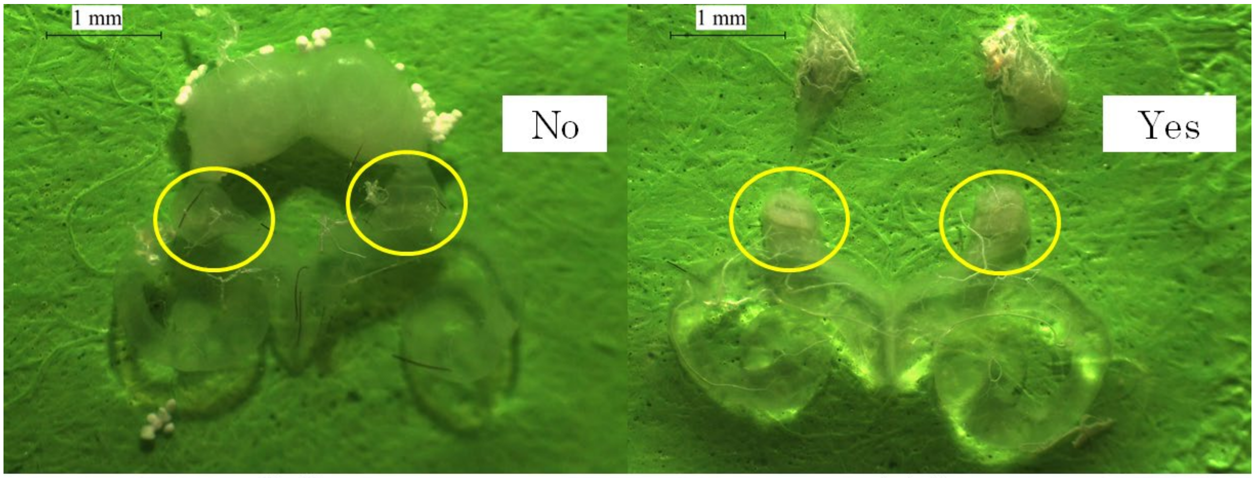
Bees were dissected to remove the MIRA for measurement. Males were briefly chilled, then pinned ventral side up to foam board coated with parafilm. I removed the sternites and gut tissue to expose the reproductive apparatus. The MIRA was then removed from the metasomal cavity by cutting the base of the ejaculatory duct and placing the intact apparatus in a bath of insect Ringer's solution ventral side-up on a green background for contrast. Images were taken using a Leica dissecting microscope and the Leica LAS v4.5 imaging software. The Leica LAS v4.5 software also placed a scale bar on each photo in order to measure surface area. The surface area of the testes and accessory testes was measured using ImageJ software (Schneider et al 2012). The total length of the accessory glands (sum of left and right glands), testes area, and accessory testes area were measured for each individual. A testes ratio was calculated as the total testes area divided by the total accessory testes area (Figure 3-2A). Thus the ratio was inversely proportional to maturation of the MIRA. The change in the ratio was used to quantify the change of the testes surface area and the accessory testes area over time and to quantify the change occurring in the testes size. I visually assessed the opacity of the accessory testes and coverage of trachea on the scrotal membrane of the testes (Figure 3-2B). Presence of sperm was observable by the presence of opaque rather than translucent accessory testes. Changes in accessory glands as bees aged were assessed by measuring the sum of the length of both accessory glands (Figure 3-2C). A high concentration of trachea due to the diminishment of the testes was scored visually by whether or not more than half of the testes surface area is covered by trachea (Figure 3-2D).

To assess body size of the individual bees, I measured the marginal cell length of the right forewing of each dissected male with Leica LAS v4.5 software under a dissecting stereoscope (Owen, 1989). Body size has been shown to affect spermatozoa counts in other corbiculate bees and marginal cell length is known to accurately reflect overall body mass in bees (Owen, 1989). Wings were removed from each bee and adhered to a glass slide with transparent tape to flatten the wing for more accurate measurements. I was blinded to the age, colony source, and marginal wing cell measurements by assigning new IDs during all dissections and measurements (Fig 3-3).

A)

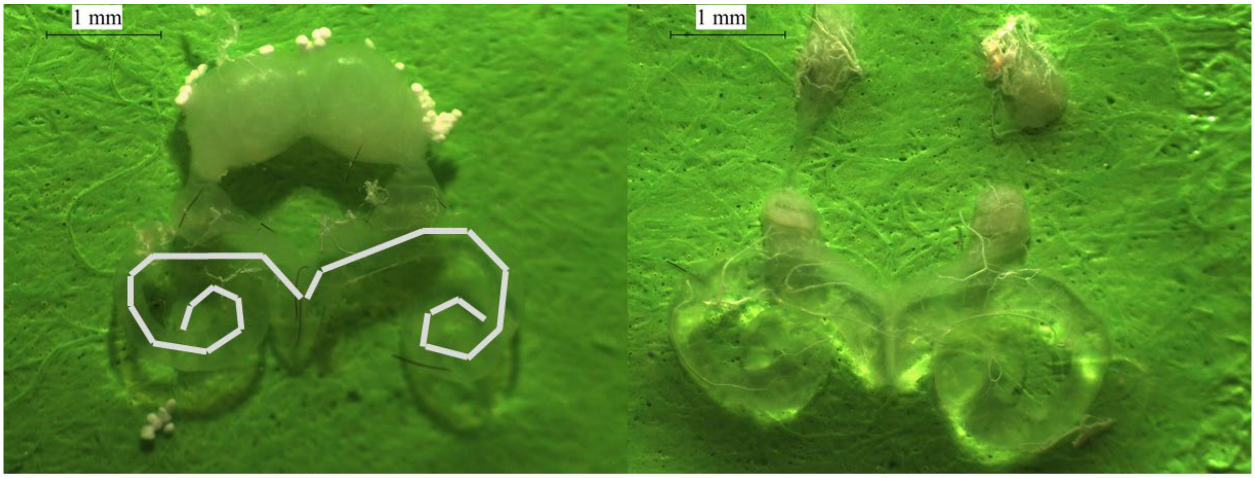


B)



Callow 14 days

C)



Callow 14 days

D)

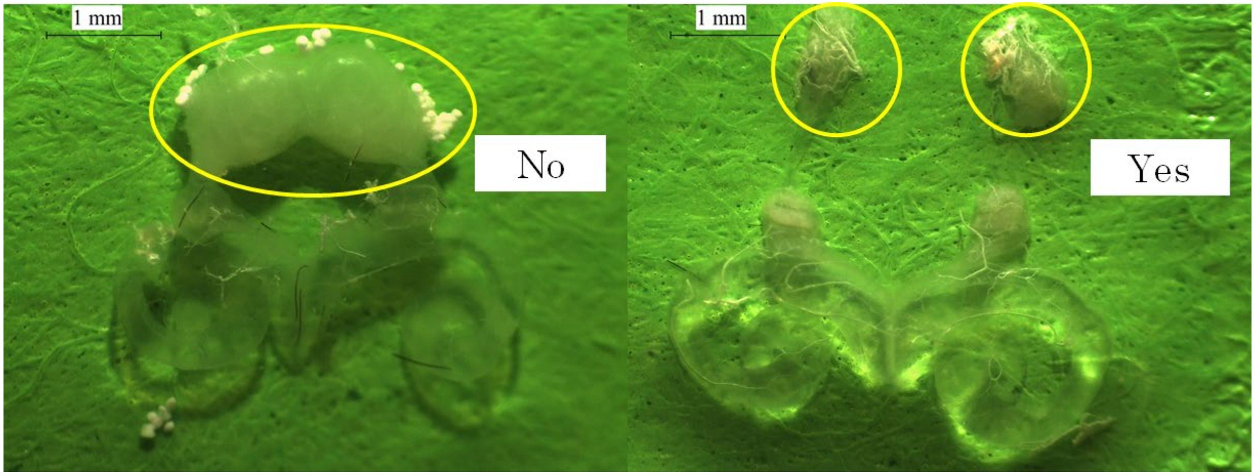


Figure 3-2. Measurements of *B. vosnesenskii* MIRA. (A) The testes ratio measurements and how the value is calculated. The gray shaded area represents the testes surface area measured and the light blue is the area of the accessory testes measured. These measurements were used to create the teste ratio illustrated on the right (B) Observation of the accessory testes storing mature sperm and scoring as a binomial response. The yellow circles show the accessory testes that are being quantified in a callow male (left) and a 14 day old male (right). Opaqueness is observed in the callow male, but not in the 14 day old male (C) An example of accessory gland length measured in a callow male and a 14 day old bee (D) Scoring the testes as being covered in trachea as a binomial response: Callow male (left) most of the surface area is not covered with condensed trachea and a 14 day old male (right) with a high concentration of trachea on the testes.

Sperm viability assay

I assessed sperm viability within the accessory testes using fluorescent nucleic acid staining dyes following procedures of Tasei et al. (1998), but modifying the procedure to work with the SYBR14 Live/Dead sperm kit™. I used queen-right males ages 4, 7, and 10 days to qualitatively assess live sperm availability at different ages post-eclosion. I first made a fluorescence working solution containing both SYBR 14 and propidium iodide from an Invitrogen™ LIVE/DEAD™ Sperm Viability Kit. In one 2.5 mL vial, I added 2 µL of SYBR 14 with 98 µL of insect Ringer's solution and another solution of 10 mL of propidium iodide mixed with 90 mL of insect Ringer's. I then placed 25 µL of the working solution in a 2.5 mL tube to be mixed with extracted sperm. I placed a 25 µL drop of insect Ringer's solution on the dissecting platform covered with a layer of fresh parafilm for extracting sperm within. After removing the guts, Malpighian tubules, and ganglia, I used two forceps to remove each of the accessory testes by holding the apical and basal end of the accessory testes so as not to lose sperm in the metasomal cavity. The sperm was extracted by pulling apart the accessory testes with the forceps inside of the 25 µL drop of Ringer's solution on the dissecting platform. When the accessory testes pull apart, the sperm could be viewed leaving the accessory testes quickly within the 25 µL of insect Ringer's solution on the dissecting platform. The ~25 µL solution of sperm and insect Ringer's solution was then added to the 25 µL of working solution of fluorescent dyes placed in the 2.5 mL vial of working solution. After a 10 min incubation at 37 °C in a water bath, the solution was added to a Fuchs-Rosenthal hemocytometer. Slides were viewed under a green filter and a red filter sequentially to reflect green for living and red for dead sperm (Figure 3-4). All sperm appeared green whether viable or not during the count under the green filter and all dead sperm appeared red under the red filter. Viable sperm counts were then determined by the difference of the green sperm counted on the green filter to the red sperm counted on the red filter.

Figure 3-3. Measurement of the marginal cell for measuring the body size of the bees. The yellow line is an example of how the marginal cell length is recorded (Owen 1989)

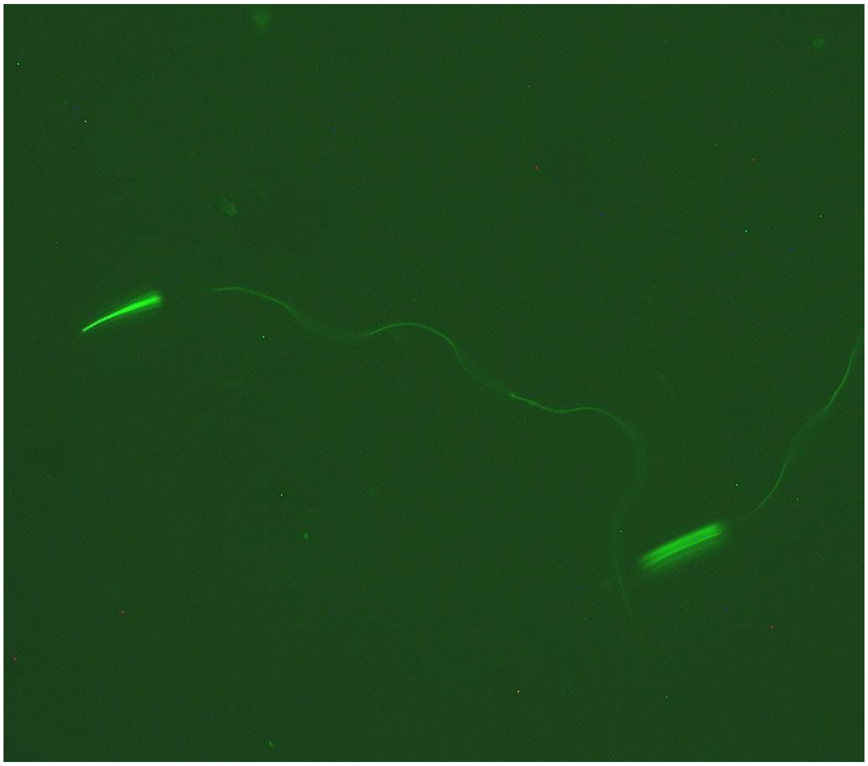


Figure 3-4. *Bombus vosnesenskii* sperm dyed with SYBR 14 under a green filter.

Statistical analysis:

I used generalized linear mixed models (glmm) to model the testes ratio and accessory gland length as a function of age, body size, colony type (queen-right or microcolony), and colony of origin (modeled as a random effect) using the glmer function in the lme4 package in R version 3.6.1 (Bates, Mächler, Bolker, & Walker, 2015). Testes ratio and accessory gland length were log-transformed to account for departures from normality. I used a generalized linear mixed model with a binomial distribution and logit link function to model the probability of opaque accessory testes and trachea covering most of the testes surface area as a function of age, colony type, and body size using the glmer function in the lme4 package. Colony of origin was included as a random effect in generalized linear mixed models.

Results:

Testes ratio diminishes with age during the first week of adulthood

Age and body size, but not colony type, were significant predictors of testes ratio (F = 52.35; N = 101; $p < 0.001$). Testes ratio significantly decreased with increasing age (t = -11.51; $p < 0.001$), indicating that the size of the testes decreased relative to the accessory testes. Bees that were older than 8 days had a testes ratio close to a value of 1, indicating that testes had shrunk to about the same size as the accessory testes (Figure 3-5). Change in both structures occurred with age, but testes surface area had a larger influence on the decreasing testes ratio than value the accessory testes. Larger males tended to have a larger testes ratio, independent of age (t = 2.00; p = 0.048). There was not a significant difference in colony type (microcolonies vs. queen-right) (t = -0.745; p = 0.458), and these types showed a similar trend in testes ratio compared with age.

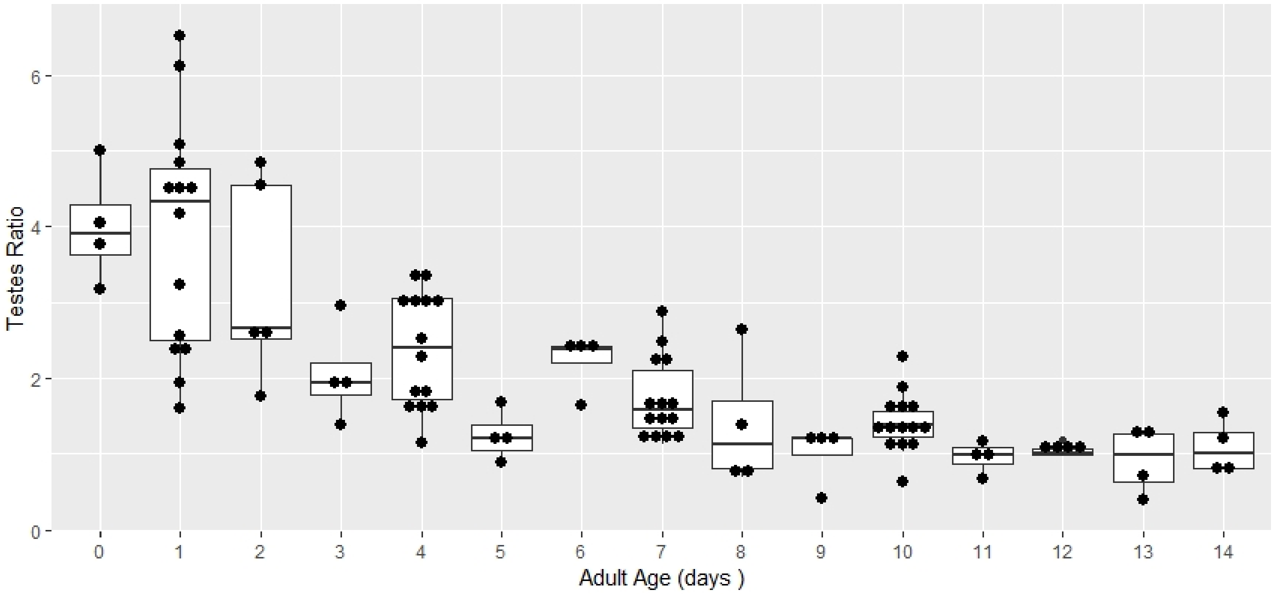


Figure 3-5. The testes ratio value compared with age across all bees sampled in a dot box plot graph. Dots are individual values; the box represents the interquartile range of testes ratio values for each age group (the range between the 25% quartile and the 75% quartile). The upper whisker of each box is the maximum value of the data that is within 1.5 times the interquartile range over the 75th percentile while the lower whisker is the minimum value of the data that is within 1.5 times the interquartile range under the 25th percentile. Any data points above the upper whisker and any points below the lower whisker are outlying testes ratio values.

Body Size, but not age, affects the accessory gland length

Body size, but not age or colony type, was a significant predictor of the length of the accessory glands. ($F = 17.88$; $N = 101$; $t = p < 0.001$). Larger bees had longer accessory glands ($t = 5.66$; $p < 0.001$; Figure 3-6). There was no significant difference in the length of the accessory glands among colony types (microcolony vs queen-right) ($t = 0.74$; $p = 0.463$).

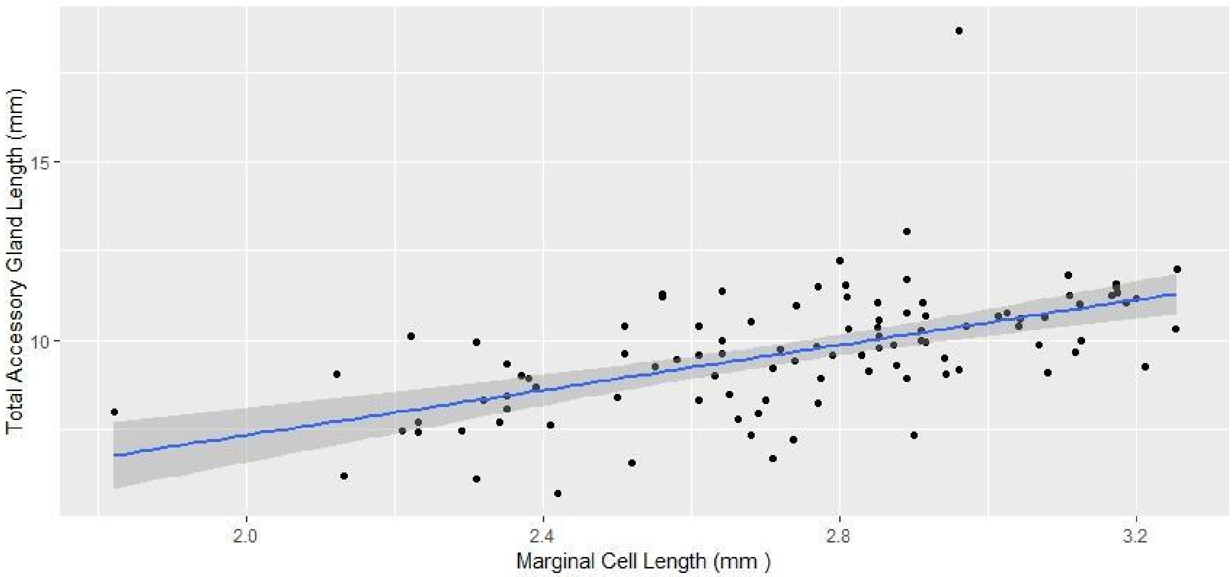


Figure 3-6. The relationship between the total accessory gland length and the marginal cell length. The length of the forewing's marginal cell is a proxy for body size (Owen 1989). The blue line shows the fitted prediction of the relationship between accessory gland length and body size, based on the glmm. The shaded region shows the 95% confidence interval along the trend line of the accessory gland length as the bees increase in size.

8-day old and older bees are most likely to have opaque testes.

Both age and body size were significant positive predictors of accessory testes opacity ($\chi^2 = 55.043$; N = 101; $p < 0.001$). Opaque accessory testes indicating mature sperm were visibly present in a larger percentage of older males than younger males ($z = 4.014$; $p < 0.001$) and all males had visible sperm in the accessory testes at day 8 and older (Fig. 3-7). Larger bees were more likely to have opaque accessory testes ($z = 2.421$;

p = 0.016). Colony type showed no significant relationship to sperm presence (z = 0.815; p = 0.415).

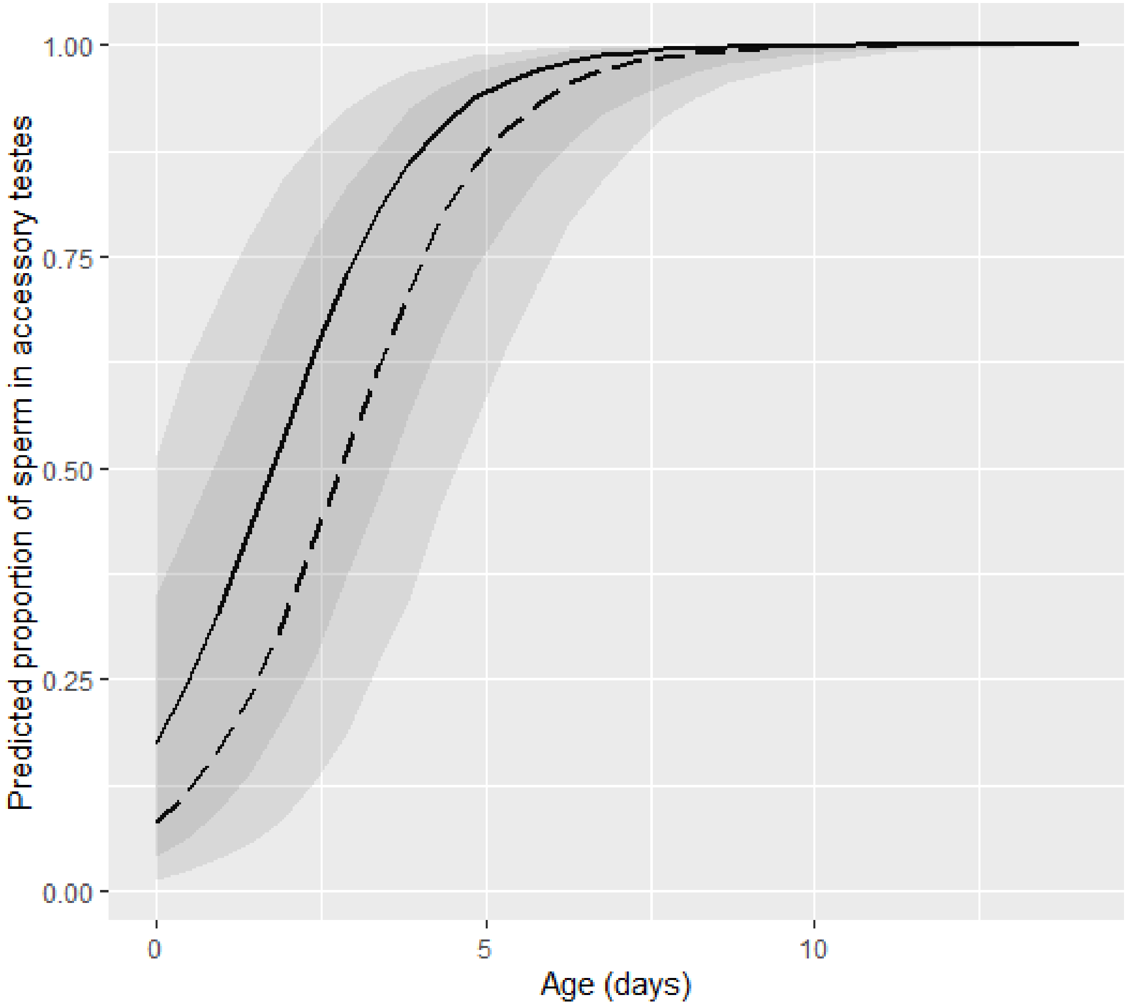


Figure 3-7. The predicted proportion of bees sampled that contain mature sperm in the accessory testes compared with age. The solid line represents microcolony males and the dashed line represents the queen-right males. The shaded portion of the graph represents the standard error of the model for each type of colony that the males came from with the darker gray being an overlap in standard error

Older males are more likely to have trachea covering the scrotal membrane surface area

Both age and body size were significant positive predictors of whether bees had trachea covering most of the scrotal membrane surface area of the testes ($\chi^2 = 58.587$; N = 101; $p < 0.001$). Older bees were more likely to have trachea covering most of the scrotal membrane surface area than younger bees ($z = 5.339$; $p < 0.001$; Fig. 3-8). Larger bees were more likely to have trachea covering most of the scrotal membrane surface area ($z = 0.968$; $p = 0.333$). Colony type was not a significant predictor of trachea on the scrotal membrane, and each colony type followed a similar pattern with increasing age ($z = 1.614$; $p = 0.106$).

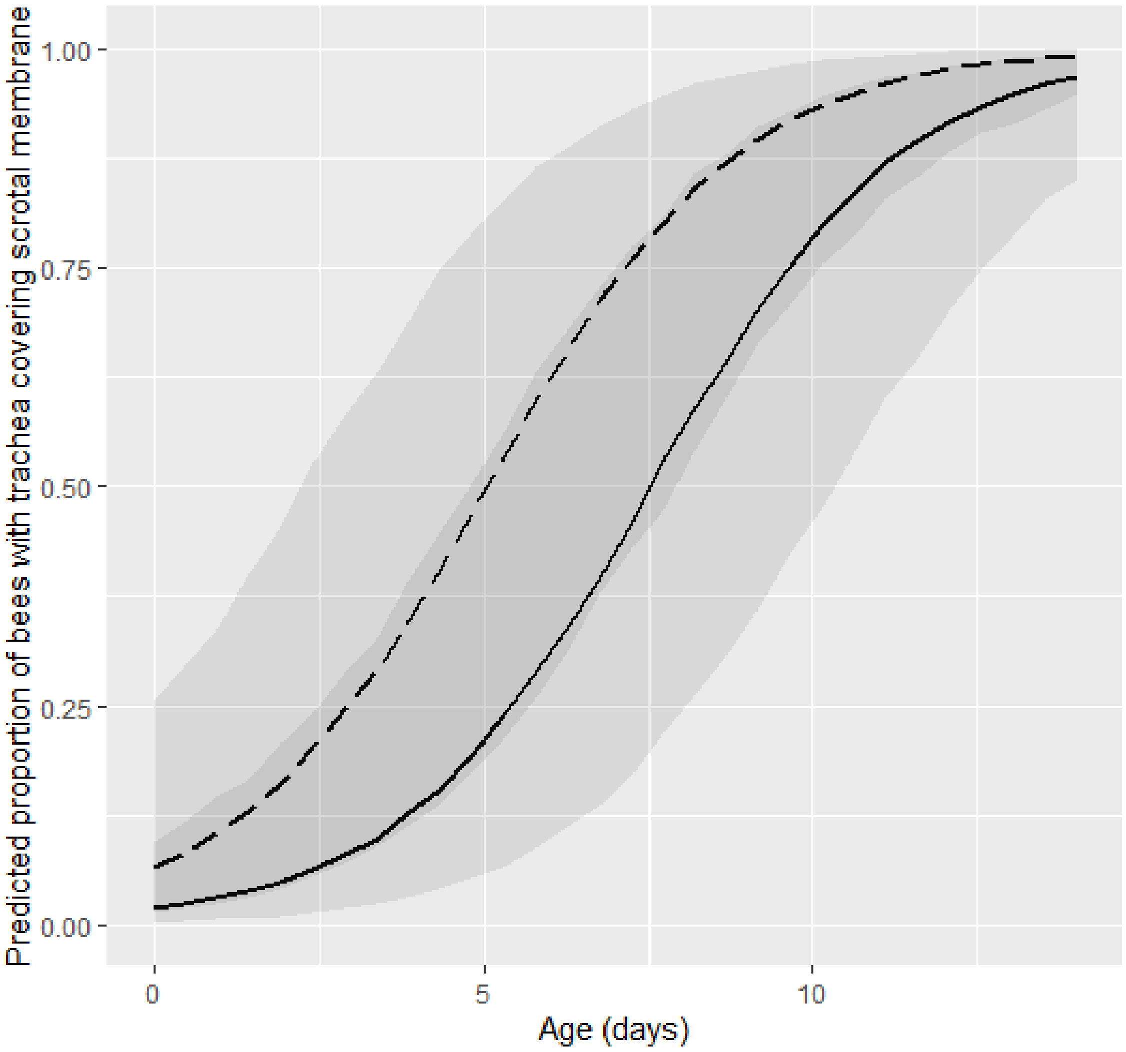


Figure 3-8. The predicted proportion of bees with surface area of the testes covered in trachea compared with age of male bees. The solid line represents microcolony males and the dashed line represents the queen-right males. The shaded portion of the graph represents the standard error of the model for each type of colony that the males came from with the darker gray being an overlap in standard error

Estimated viability and sperm counts observed in accessory testes

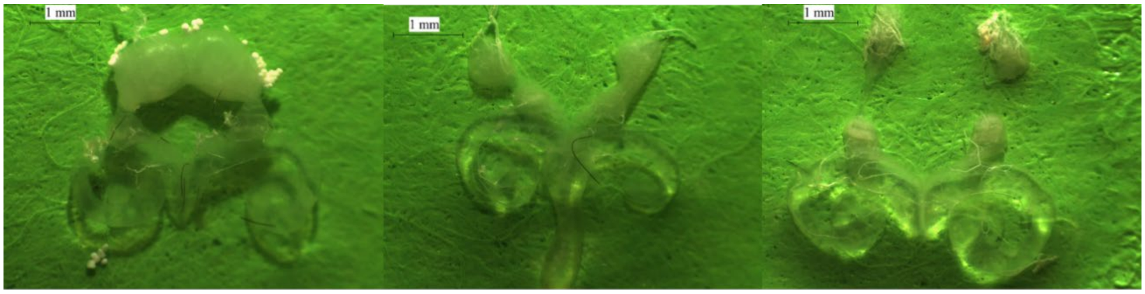
The assessment of sperm viability suggests that sperm counts and viability were relatively stable as males mature (Table 3-1). Sperm viability ranged between 79% and 84% in 4-10 days old males, except for one 7-day old outlier with 56.5% viability. Small sample size did not allow for a statistical analysis of sperm count and viability. All males sampled were from queen-right colonies.

Table 3-1

Data on sperm count and sperm viability (N = 8 bees). Includes calculated percentage of viable sperm for each sample, total number of viable sperm in the accessory testes.

Age of bee (Days)	Sperm counted on hemocytometer	Dead sperm counted on hemocytometer	Live sperm on hemocytometer	viability %	Estimated total viable sperm of extracted sample	Total viable sperm of bee
4	335	63	272	81.3	15,300	1,530,000
4	450	70	380	84.4	21,375	2,137,500
7	409	73	336	82.2	18,900	1,890,000
7	428	69	359	84.0	20,194	2,019,375
7	600	106	494	82.3	27,788	2,778,750
7	177	77	100	56.5	5,625	562,500
10	473	99	374	79.1	21,038	2,103,750
10	433	79	354	81.8	19,913	1,991,250

Overall the data show that changes in the reproductive apparatus occur primarily within 8 days where the testes have diminished in surface area to approximately the size of the accessory testes and the point that 100% of males sampled had opaque accessory testes indicating spermatozoa. Figure 3-9 shows images of three exemplary age group samples to illustrate the differences in the morphology of the MIRA of *B. vosnesenskii*. These age groups seem to be the points at which major changes occur or stabilize.



Callow   7 days post eclosion   14 days post eclosion

Figure 3-9. *B. vosnesenskii* MIRA removed at the day of eclosion (callow), 7 days post-eclosion and at 14 days post eclosion showing the observed changes in the MIRA from eclosion to sexual maturity. This figure illustrates the change that occurs in the MIRA of *B. vosnesenskii*.

Discussion

This study provides the first quantification of the post-eclosion development of the MIRA in bumble bees. This is important for optimizing the timing of mating between captive born bumble bee males and gynes for continuously production of commercial colonies. Additionally, it is important to detect differences in how males born to workers or queens mature, because generating males from workers is a commonly used practice to rapidly generate a pool of males outside of the constraints of a typical colony cycle (Strange personal communication; Velthuis & van Doorn 2006). However, males born to queens are most likely to mate with gynes in nature. Despite the importance of males in breeding, much of what is known about reproductive development in bumble bees is focused on females (Baer & Schmid-Hempel, 2000; Bloch, Hefetz, & Hartfelder, 2000; Geva, Hartfelder, & Bloch, 2005). Previous studies have described spermatogenesis in male bees at a cellular level (Cruz-Landim et al., 1980) or documented reproductive structures in bumble bees (Duchateau & Mariën, 1995; Ferreira et al., 2004). However, it is unknown how these reproductive structures change as males reach sexual maturity (Duvoisin et al., 1999; Ferreira et al., 2004). Determining optimal mating age requires an understanding of changes that occur throughout the entire male reproductive apparatus. Moreover, anatomical measurements can provide a simple heuristic for determining age of reproductive maturity that can be easily transferred to additional species. This study documents the pattern of male reproductive maturity in a species that is targeted for commercial production, but that is also easily applicable to other species.

This assessment of anatomical changes in the MIRA suggests male *B. vosnesenskii* are mature by 8 days post-eclosion. This is based on several lines of evidence. First, all of the bees in our study had opaque accessory testes by day 8. This indicates that male bumble bees 8 days or more post-eclosion have spermatozoa ready for mating with queen bumble bees. Second, I observed that the surface area of the testes diminishes up to the age of 7 days old. Samples 8 days old or older had an average testes ratio value of ~1, indicating the size of the testes was similar to the size of the accessory testes. This diminishment has been noted but has never been quantified with regard to age (Baer & Schmid-Hempel 2000; Baer 2003; Baer & Schmid-Hempel 2005; Duchateau & Mariën 1995; Ferreira et al., 2004; Greeff & Schmid-Hempel 2008; Tasei et al., 1998).

The proportion of bees with trachea covering most of the scrotal membrane surface area increased with age, and nearly all males had higher concentrations of trachea on the testes surface by 14 days of age. This suggests that the large concentrations of trachea found on the bee's testes are a result of the diminishment of the size of the testes that comes with age. The more gradual slope of predictive probability that the scrotal membrane of the testes will be predominately covered in traches indicates further degradation of the testes over time.

Adult male bumble bees show behavioral and other anatomical changes that also indicate sexual maturity occurring at 8 days post-eclosion. In *B. terrestris* males, reduction of the cephalic region of the labial gland is associated with the production of patrolling pheromones used to mark mating-flight territory of males and to attract gynes (Šobotník et al., 2008; Valterová, Martinet, Michez, Rasmont, & Brasero, 2019). Šobotník et al (2008) found that pheromone production and the main component of the marking pheromone of males (Dihydrofarnesol) increased from 1 day up to 7 days post-eclosion in *B. terrestris* (Šobotník et al., 2008). This coincides with when 100% of *B. vosnesenskii* males had mature sperm in their accessory testes and the earliest age that *B. terrestris* males showed behaviors of reproductive receptivity (Tasei et al., 1998). This may mean that *B. vosnesenskii* has a similar pattern of pheromone production and sexual maturity as *B. terrestris*. These and other data suggest that sexual maturation across the genus *Bombus* occurs in males around 8 days of age, but empirical data across the genus is lacking. Future research could investigate whether reduction of the cephalic region of the labial gland and change in pheromone production correlates with testes diminishment and sperm presence in the accessory testes across the *Bombus* genus.

Sperm counts in the males sampled were relatively stable but high compared to other bumble bee species that have had their viable sperm counted (Tasei et al., 1998; Greeff & Schmid-Hempel 2008). It is hard to draw a conclusion to why they are stable when there are obvious changes occurring during spermatogenesis and other species show a fluctuation in sperm counts later than observed with *B. vosnesenskii* (Tasei et al., 1998; Greeff & Schmid-Hempel 2008). A study comparing sperm viability of monandrous and polyandrous insect species showed a trend towards higher viability and higher sperm quality within polyandrous species than in monandrous species (Hunter & Birkhead, 2002). Hunter and Birkhead (2002) suggest that higher sperm quality and viability is a selective pressure on polyandrous species and therefore higher sperm quality between highly related species may indicate polyandrous behavior in insects due to sperm competition (Hunter & Birkhead, 2002). Although rarely observed, *B. vosnesenskii* has been shown to have some polyandrous queens in the wild and therefore these highly viable and stable sperm counts could indicate adaptations to polyandry in queens that are facultative polyandrous (Strange & Picklum, unpublished data). Another consideration is that *B. vosnesenskii* produce large colonies compared to other bumble bee species, and continually lay workers before ever laying gynes when reared in captivity (personal observation). This species may thus have unusually large quantities of sperm compared to other species because there is a large paternal investment of sperm necessary to produce large colonies. However bumble bee queens have been shown to have large quantities of sperm stored in their spermatheca even during the senescence of the colony after laying the reproductive castes. A wider age of ranges and a larger sample size would allow for more detailed analysis of patterns of viable spermatozoa counts in *B. vosnesenskii*.

The results of this study suggest that *B. vosnesenskii* males aged 8 days or older have completed sexual maturation of MIRA development, thus this is the earliest age that males should be introduced to gynes for mating. Successful sperm transfer is also contingent on the copulatory behavior of both the male and the gyne. The low number of gynes produced by this species in captivity makes it difficult to investigate the precopulatory and copulatory behaviors of *B. vosnesenskii* (Šobotník et al., 2008; Valterová et al., 2019). In *B. terrestris*, males are known to be receptive to gynes and mate by as early as 7 days old, but the average age of male copulation was 12.1 days (Tasei et al., 1998). Therefore, determining optimal age of mating for *B. vosnesenskii* may require additional metrics, such as receptivity to gynes and vice versa. Based on morphology alone though, 8 days post-eclosion is the time period at which the bees are morphologically mature.

Another method for breeding bumble bee colonies in captivity is artificial insemination (Baer & Schmid-Hempel, 2000). If *B. vosnesenskii* males require extra time to develop their sexually reproductive behaviors then this artificial insemination method may allow breeders to bypass that stage of development (Baer & Schmid-Hempel, 2000). This study is then valuable for knowing when one can extract spermatozoa from males for artificial insemination of gynes. This method of insemination is more time consuming and requires specialization in the methods presented by Baer & Schmid-Hempel (2001) but allows for more control over this portion of the colony life cycle.

I investigated if there are phenotypic differences in males produced from each colony-type and found that the process of reproductive maturation is the same for males laid from microcolonies and from queen-right colonies. This is important, because large-scale production of bumble bee colonies relies on the successful reproduction of males and gynes. This requires a large investment of time and resources to have bumble bee colonies reach maturity in order to produce reproductive castes. Commercial producers of bumble bee colonies use males generated from microcolonies to mate with gynes for continual captive lines of bumble bee colonies (Klinger et al. 2019). Male production with microcolonies reduces phenological constraints of the bumble bee lifecycle by having better control of when males are produced. The results of this study indicate no major differences in males from either colony. Therefore, utilization of worker-laid males from microcolonies may be a productive method for obtaining high quality males for mating with gynes. Besides the applied utility of males from microcolnies, it is a fascinating finding that there are no apparent differences between microcolony males and queen-right males because these 2 colony types likely also have eggs laid by the two different female castes (Owen & Plowright, 1982). Given the differences in reproductive physiology between queens and workers, one would expect that there may be differences in investment and development of the males that each female caste lays. This shows that offspring of workers is likely just as viable as offspring from queens but further research on differences between colony types should be done on sperm viability, quantity, and motility to further investigate whether there are differences between males from different colony types.

The size of the bee sampled has a significant effect on all the morphological measurements of the MIRA except for the trachea covering the scrotal membrane of males. Body size was a significant predictor of the testes ratio, which could indicate that larger bees have larger testes at eclosion and therefore may produce more sperm. In honey bees, larger males have 37% higher sperm counts (Schlüns 2003), and it is likely that the larger male bumble bees have higher sperm counts as well (Owen, 1989). The total length of the accessory glands increased as the size of the bee increased, but did not change in length as bees aged. This suggests the accessory glands are fully grown at the time of eclosion. However, there may be other post-eclosion physiological changes that occur in the accessory gland. The trend in longer accessory glands with larger body size is not surprising, but interesting because of the role that accessory glands play in preventing multiple males from mating with a gyne by producing larger mating plugs (Baer et al 2001).

Further investigation into whether larger males are able to produce larger mating plugs that may last longer in the bursa copulatrix of the gyne may provide some insight into the evolution of multiple mating (Baer et al., 2003; Brown et al., 2002; Brown & Schmid-Hempel, 2003). Differences in the mating plug size have been documented across species (Brown et al., 2002; Brown & Schmid-Hempel, 2003). The size of the mating plug can be small enough to render it ineffective in stopping other male spermatozoa from entering the spermatheca, and ineffective in inhibiting gyne receptivity to extra-male copulations (Brown & Schmid-Hempel., 2003). Colony-level differences have been previously reported for accessory gland size in *B. hypnorum* (Brown et al., 2002), but this showed no significant variation among source colonies or between colony types in *B. vosnesenskii*. This could have something to do with differences between *B. hypnorum* and *B. vosnesenskii* mating strategies. *Bombus hypnorum* and a few other species of bumble bees show some evidence of facultative polyandrous queens. Brown et al (2002) found that polyandrous colonies produced males that had smaller accessory glands implying that there may be some correlation with investment in the size of the accessory glands and the investment in the size of the mating plug (Brown & Schmid-Hempel, 2003), making it more likely to fail at inhibiting polyandry in the bumble bee queen (Brown et al., 2002). Interspecific variation in bumble accessory gland length could indicate a bumble bee species' likelihood of multiple mating in nature as it has been observed.

Conclusion:

The MIRA of *B. vosnesenskii* undergo major morphological changes up to the age of 8 days post eclosion. These changes occur in the testes and accessory testes with spermatozoa presence in the accessory testes and the total surface area of the testes diminishing to about the size of the accessory testes occurring in male *B. vosnesenskii*. There is evidence of continual degradation of the testes beyond this age as well with more samples showing high concentrations of trachea as the males get older. Accessory gland length was not affected by the age of the bee indicating accessory gland development is completed pre-eclosion. All metrics of the morphological changes were significantly affected by the size of the bee with larger bees having a larger testes ratio, sperm more likely to be in the accessory glands, and longer accessory glands. This information can now help to understand basic biological processes occurring in adult male bumble bees.

This study is a needed step to understand the reproductive development of male bumble bees. Because this study is the first to quantify these developmental changes occurring in any species of bumble bees, it will not only help to understand the reproductive development of bumble bees with an applied usage but it also serves as a baseline for any bumble bee species. This opens opportunities into understanding other components of male bumble bee development and the effects of biotic and abiotic factors that may influence male bumble bee development. Now that the external morphology of bumble bee pupae has been quantified (Tian & Hines, 2018), it would then be of value to track these changes within the pupae because this is the life stage at which most of the MIRA development takes place (Ferreira et al., 2004).

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Literature cited:

Aizen, M. A., Smith-Ramírez, C., Morales, C. L., Vieli, L., Sáez, A., Barahona-Segovia, R. M., … Harder, L. D. (2018). Coordinated species importation policies are needed to reduce serious invasions globally: The case of alien bumblebees in South America. *Journal of Applied Ecology*, *56*(1), 100–106. https://doi.org/10.1111/1365-2664.13121

Alaux, C., Boutot, M., Jaisson, P., & Hefetz, A. (2007). Reproductive Plasticity in Bumblebee Workers (*Bombus terrestris*): Reversion from Fertility to Sterility under Queen Influence. *Behavioral Ecology and Sociobiology*, *62*(2), 213–222. https://doi.org/10.1007/s00265-007-0455-6

Alford, D. V. (1975). *Bumblebees*. London: Davis-Poynter.

Artz, D. R., & Nault, B. A. (2011). Performance of *Apis mellifera*, *Bombus impatiens*, and *Peponapis pruinosa* (Hymenoptera: Apidae) as Pollinators of Pumpkin. *Journal of Economic Entomology*, *104*(4), 1153–1161. https://doi.org/10.1603/ec10431

Baer, B. (2003). Bumblebees as model organisms to study male sexual selection in social insects. *Behavioral Ecology and Sociobiology*, *54*(6), 521–533. https://doi.org/10.1007/s00265-003-0673-5

Baer, B., Morgan, E. D., & Schmid-Hempel, P. (2001). A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(7), 3926–3928. https://doi.org/10.1073/pnas.061027998

Baer, B., & Schmid-Hempel, P. (2000). The artificial insemination of bumblebee queens. *Insectes Sociaux*, *47*(2), 183–187. https://doi.org/10.1007/PL00001699

Baer, B., & Schmid-Hempel, P. (2005). Sperm influences female hibernation success, survival and fitness in the bumblebee *Bombus terrestris*. *Proceedings. Biological Sciences / The Royal Society*, *272*(1560), 319–323. https://doi.org/10.1098/rspb.2004.2958

Baer, B., Schmid-Hempel, P., Høeg, J. T., & Boomsma, J. J. (2003). Sperm length, sperm storage and mating system characteristics in bumblebees. *Insectes Sociaux*, *50*(2), 101–108. https://doi.org/10.1007/s00040-003-0641-0

Banda, H. J., & Paxton, R. J. (1991). Pollination of Greenhouse Tomatoes by Bees. *Acta Horticulturae*, (288), 194–198. https://doi.org/10.17660/ActaHortic.1991.288.28

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1). https://doi.org/10.18637/jss.v067.i01

Bloch, G., Hefetz, A., & Hartfelder, K. (2000). Ecdysteroid titer, ovary status, and dominance in adult worker and queen bumble bees (*Bombus terrestris*). *Journal of Insect Physiology*, *46*(6), 1033–1040. https://doi.org/https://doi.org/10.1016/S0022-1910(99)00214-0

Brown, M. J. F., Baer, B., Schmid-Hempel, R., & Schmid-Hempel, P. (2002). Dynamics of multiple-mating in the bumble bee *Bombus hypnorum*. *Insectes Sociaux*, *49*(4), 315–319. https://doi.org/10.1007/PL00012654

Brown, M. J. F., & Schmid-Hempel, P. (2003). The Evolution Of Female Multiple Mating in Social Hymenoptera. *Ecology and Evolutiony and Evolution*, *57*(9), 2067–2081. https://doi.org/10.1111/j.0014-3820.2003.tb00386.x

Buttermore, R. E., Pomeroy, N., Hobson, W., Semmens, T., & Hart, R. (1998). Assessment of the genetic base of Tasmanian bumble bees (*Bombus terrestris*) for development as pollination agents. *Journal of Apicultural Research*, *37*(1), 23–25. https://doi.org/10.1080/00218839.1998.11100950

Byatt, M. A., Chapman, N. C., Latty, T., & Oldroyd, B. P. (2016). The genetic consequences of the anthropogenic movement of social bees. *Insectes Sociaux*, Vol. 63, pp. 15–24. https://doi.org/10.1007/s00040-015-0441-3

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., & Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, *108*(2), 662–667. https://doi.org/10.1073/pnas.1014743108

Cameron, Sydney A, Lim, H. C., Lozier, J. D., Duennes, M. A., & Thorp, R. (2016). Test of the invasive pathogen hypothesis of bumble bee decline in North America. *Proceedings of the National Academy of Sciences*, *113*(16), 4386–4391. https://doi.org/10.1073/pnas.1525266113

Cnaani, J., Schmid-Hempel, R., & Schmidt, J. O. (2002). Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson. *Insectes Sociaux*, *49*(2), 164–170. https://doi.org/10.1007/s00040-002-8297-8

Cruz-Landim, C., Beig, D., & Silva De Moraes, R. L. M. (1980). The process of differentiation during spermatogenesis in bees (Hymenoptera: Apidae). *Caryologia*, *33*(1), 1–15. https://doi.org/10.1080/00087114.1980.10796814

Desjardins, È.-C., & De Oliveira, D. (2009). Commercial Bumble Bee *Bombus impatiens* (Hymenoptera: Apidae) as a Pollinator in Lowbush Blueberry (Ericale: Ericaceae) Fields. *Journal of Economic Entomology*, *99*(2), 443–449. https://doi.org/10.1603/0022-0493-99.2.443

Dogterom, M. H., Matteoni, J. A., & Plowright, R. C. (1998). Pollination of Greenhouse Tomatoes by the North American *Bombus vosnesenskii* (Hymenoptera: Apidae). *Journal of Economic Entomology*, *91*(1), 71–75. https://doi.org/10.1093/jee/91.1.71

Donovan, B. J., & Wier, S. S. (1984). Development of hives for field population increase, and studies on the life cycles of the four species of introduced bumble bees in New Zealand. *New Zealand Journal of Agricultural Research*, *21*(4), 733–756. https://doi.org/10.1080/00288233.1978.10427476

Duchateau, M. J., & Mariën, J. (1995). Sexual biology of haploid and diploid males in the bumble bee *Bombus terrestris*. *Insectes Sociaux*, *42*(3), 255–266.

https://doi.org/10.1007/BF01240420

Duchateau, M. J., & Velthuis, H. H. W. (1989). Ovarian development and egg laying in workers of *Bombus terrestris*. *Entomologia Experimentalis et Applicata*, *51*(3), 199–213. https://doi.org/10.1111/j.1570-7458.1989.tb01231.x

Duvoisin, N., Baer, B., & Schmid-Hempel, P. (1999). Sperm transfer and male competition in a bumblebee. *Animal Behaviour*, *58*(4), 743–749. https://doi.org/10.1006/anbe.1999.1196

Evans, E., Burns, I., & Spivak, M. (2007). *Befriending bumble bees* University of Minnesota Extension

Ferreira, A., Abdalla, F. C., Kerr, W. E., & Cruz-Landim, C. da. (2004). Comparative anatomy of the male reproductive internal organs of 51 species of bees. *Neotropical Entomology*, *33*(5), 569–576. Retrieved from http://dx.doi.org/10.1590/S1519-566X2004000500005

Geva, S., Hartfelder, K., & Bloch, G. (2005). Reproductive division of labor, dominance, and ecdysteroid levels in hemolymph and ovary of the bumble bee Bombus terrestris. *Journal of Insect Physiology*, *51*(7), 811–823. https://doi.org/10.1016/j.jinsphys.2005.03.009

Goka, K., Okabe, K., Yoneda, M., & Niwa, S. (2001). Bumblebee commercialization will cause worldwide migration of parasitic mites. *Molecular Ecology*, *10*(8), 2095–2099. https://doi.org/10.1046/j.0962-1083.2001.01323.x

Goulson, D. 2010. *Bumblebees: Behaviour, ecology, and conservation*. Oxford: Oxford University Press

Graystock, P., Yates, K., Evison, S. E. F., Darvill, B., Goulson, D., & Hughes, W. O. H. (2013). The Trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies. *Journal of Applied Ecology*, *50*(5), 1207–1215. https://doi.org/10.1111/1365-2664.12134

Greeff, M., & Schmid-Hempel, P. (2008). Sperm viability in the male accessory testes and female spermathecae of the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). *European Journal of Entomology*, *105*(5), 849–854. https://doi.org/10.14411/eje.2008.112

Hunter, F. M., & Birkhead, T. R. (2002). Sperm viability and sperm competition in insects. *Current Biology*, *12*(2), 121–123. https://doi.org/10.1016/S0960-9822(01)00647-9

Huth-Schwarz, A., León, A., Vandame, R., Moritz, R. F. A., & Kraus, F. B. (2011). Workers dominate male production in the neotropical bumblebee *Bombus wilmattae* (Hymenoptera: Apidae). *Frontiers in Zoology*, *8*(1), 13. https://doi.org/10.1186/1742-9994-8-13

Ings, T. C., Ward, N. L., & Chittka, L. (2006). Can commercially imported bumble bees out-compete their native conspecifics? *Journal of Applied Ecology*, *43*(5), 940–948. https://doi.org/10.1111/j.1365-2664.2006.01199.x

Jackson, J. M., Pimsler, M. L., Oyen, K. J., Koch-Uhuad, J. B., Herndon, J. D., Strange, J. P., … Lozier, J. D. (2018). Distance, elevation and environment as drivers of diversity and divergence in bumble bees across latitude and altitude. *Molecular Ecology*, *27*(14), 2926–2942. https://doi.org/10.1111/mec.14735

Kanbe, Y., Okada, I., Yoneda, M., Goka, K., & Tsuchida, K. (2008). Interspecific mating of the introduced bumblebee *Bombus terrestris* and the native Japanese bumblebee *Bombus hypocrita sapporoensis* results in inviable hybrids. *Naturwissenschaften*, *95*(10), 1003–1008. https://doi.org/10.1007/s00114-008-0415-7

Klinger, E. G., Camp, A. A., Strange, J. P., Cox-Foster, D., & Lehmann, D. M. (2019). *Bombus* (Hymenoptera: Apidae) Microcolonies as a Tool for Biological Understanding and Pesticide Risk Assessment. *Environmental Entomology*. https://doi.org/10.1093/ee/nvz117

Korner, P., & Schmid-Hempel, P. (2003). Effects of sperm on female longevity in the bumblebee *Bombus terrestris*. *Proceedings of the Royal Society B: Biological Sciences*, *270*(2), 227–229. https://doi.org/10.1098/rsbl.2003.0039

Lensing, C. (2018). *Controlled Environment Agriculture : Farming for the Future ?* (May), 1–7.

Liberti, J., Görner, J., Welch, M., Dosselli, R., Schiøtt, M., Ogawa, Y., Baer, B. (2019). Seminal fluid compromises visual perception in honeybee queens reducing their survival during additional mating flights. *ELife*, *8*, 1–27. https://doi.org/10.7554/elife.45009

Looney, C., Strange, J. P., Freeman, M., Jennings, D., & Jennings, D. (2019). The expanding Pacific Northwest range of *Bombus impatiens* Cresson and its establishment in Washington State. *Biological Invasions*, *21*(6), 1879–1885. https://doi.org/10.1007/s10530-019-01970-6

Macfarlane, R. P., & Gurr, L. (1995). Distribution of bumble bees in new zealand. *New Zealand Entomologist*, *18*(1), 29–36. https://doi.org/10.1080/00779962.1995.9721999

Malfi, R. L., Crone, E., & Williams, N. (2019). Demographic benefits of early season resources for bumble bee (*B. vosnesenskii*) colonies. *Oecologia*, *191*(2), 377–388. https://doi.org/10.1007/s00442-019-04472-3

Meeus, I., Brown, M. J. F., De Graaf, D. C., & Smagghe, G. (2011). Effects of Invasive Parasites on Bumble Bee Declines. *Conservation Biology*, Vol. 25, pp. 662–671. https://doi.org/10.1111/j.1523-1739.2011.01707.x

Mikheyev, A. S. (2004). Male accessory gland size and the evolutionary transition from single to multiple mating in the fungus-gardening ants. *Journal of Insect Science*, *4*(37), 1–5. https://doi.org/10.1673/031.004.3701

Morales, C. L., Arbetman, M. P., Cameron, S. A., & Aizen, M. A. (2013). Rapid ecological replacement of a native bumble bee by invasive species. *Frontiers in Ecology and the Environment*, *11*(10), 529–534. https://doi.org/10.1890/120321

Morandin, L. A., Laverty, T. M., & Kevan, P. G. (2001). Bumble Bee (Hymenoptera: Apidae) Activity and Pollination Levels in Commercial Tomato Greenhouses. *Journal of Economic Entomology*, *94*(2), 462–467. https://doi.org/10.1603/0022-0493-94.2.462

Murray, T. E., Coffey, M. F., Kehoe, E., & Horgan, F. G. (2013). Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. *Biological Conservation*, *159*, 269–276. https://doi.org/10.1016/j.biocon.2012.10.021

Otterstatter, M. C., & Thomson, J. D. (2008). Does Pathogen Spillover from Commercially Reared Bumble Bees Threaten Wild Pollinators? *PLOS ONE*, *3*(7), 1–9. https://doi.org/10.1371/journal.pone.0002771

Owen, R. E., & Plowright, R. C. (1982). Worker-Queen Conflict and Male Parentage in Bumble Bees. *Behavioral Ecology and Sociobiology*, *11*(2), 91–99.

Owen, R. E. (1989). Differential Size Variation of Male and Female Bumblebees. *Journal of Heredity*, *80*(1), 39–43. https://doi.org/10.1093/oxfordjournals.jhered.a110786

Rao, S., & Stephen, W. P. (2007). *Bombus* (*Bombus*) *occidentalis* (Hymenoptera: Apiformes): In decline or recovery. *The Pan-Pacific Entomologist*, *83*(4), 360–362. https://doi.org/10.3956/2007-10.1

Regali, A., & Rasmont, P. (1995). Nouvelles méthodes de test pour l'évaluation du régime alimentaire chez des colonies orphelines de *Bombus terrestris* (L) (Hymenoptera, Apidae). *Apidologie*, *26*(4), 273–281. https://doi.org/10.1051/apido:19950401

Sachman-Ruiz, B., Narváez-Padilla, V., & Reynaud, E. (2015). Commercial *Bombus impatiens* as reservoirs of emerging infectious diseases in central México. *Biological Invasions*, *17*(7), 2043–2053. https://doi.org/10.1007/s10530-015-0859-6

Sapir, G., Goldway, M., & Stern, R. A. (2019). Supplementing bumblebees to 'Mauritius' lychee improves yield. *Scientia Horticulturae*, *251*, 162–166. https://doi.org/https://doi.org/10.1016/j.scienta.2019.03.015

Schneider, C.A., Rasband, W.S., Eliceiri, K.W. "NIH Image to ImageJ: 25 years of image analysis". Nature Methods 9, 671-675, 2012.

Schlüns, H., Schlüns E., van Praagh, E., Moritz, R., Sperm numbers in drone honey-bees (Apis mellifera) depend on body size. Apidologie, Springer Verlag, 2003, 34 (6), pp.577-584. http:doi.org10.1051/apido:2003051

Sladen, F. 1912. *The Humble-Bee: Its Life-History and How to Domesticate it, with Descriptions of All the British Species of Bombus and Psithyrus* (Cambridge Library Collection - Zoology). Cambridge: Cambridge University Press. doi:10.1017/CBO9781107705739

Snodgrass, R. E. (1910). Anatomy of the Honey Bee. In *Anatomy of the Honey Bee*.

https://doi.org/10.7591/9781501717925

Šobotník, J., Kalinová, B., Cahlíková, L., Weyda, F., Ptáček, V., & Valterová, I. (2008). Age-dependent changes in structure and function of the male labial gland in *Bombus terrestris*. *Journal of Insect Physiology*, *54*(1), 204–214. https://doi.org/https://doi.org/10.1016/j.jinsphys.2007.09.003

Stout, J. C., & Goulson, D. (2000). Bumble bees in Tasmania: their distribution and potential impact on Australian flora and fauna. *Bee World*, *81*(2), 80–86. https://doi.org/10.1080/0005772X.2000.11099475

Stout, J. C., & Morales, C. L. (2009). Ecological impacts of invasive alien species on bees. *Apidologie*, *40*(3), 388–409. https://doi.org/10.1051/apido/2009023

Strange, J. P. (2010). Nest Initiation in Three North American Bumble Bees (*Bombus*): Gyne Number and Presence of Honey Bee Workers Influence Establishment Success and Colony Size. *Journal of Insect Science*, *10*(130), 1–11. https://doi.org/10.1673/031.010.13001

Strange, J. P. (2015). *Bombus huntii*, *Bombus impatiens*, and *Bombus vosnesenskii* (Hymenoptera: Apidae) Pollinate Greenhouse-Grown Tomatoes in Western North America. *Journal of Economic Entomology*, *108*(3), 873–879. https://doi.org/10.1093/jee/tov078

Stubbs, C. S., & Drummond, F. A. (2001). *Bombus impatiens* (Hymenoptera : Apidae): An Alternative to *Apis mellifera* (Hymenoptera : Apidae) for Lowbush Blueberry Pollination. *Apiculture and Social Insects*, *94*(3), 609–616. Retrieved from https://doi.org/10.1603/0022-0493-94.3.609

Sutherland, W. J., Barnard, P., Broad, S., Clout, M., Connor, B., Côté, I. M., … Ockendon, N. (2017). Feature Review A 2017 Horizon Scan of Emerging Issues for Global Conservation and Biological Diversity. *Trends in Ecology & Evolution*, *32*(1), 31–40. https://doi.org/10.1016/j.tree.2016.11.005

Tasei, J. N., Moinard, C., Moreau, L., Himpens, B., & Guyonnaud, S. (1998). Relationship between aging, mating and sperm production in captive *Bombus terrestris*. *Journal of Apicultural Research*, *37*(2), 107–113. https://doi.org/10.1080/00218839.1998.11100962

Thornsbury, S., Wells, H., & Bond, J. (2016). Vegetables and pulses outlook. In *Change* (Vol. 2015).

Tian, L., & Hines, H. M. (2018). Morphological characterization and staging of bumble bee pupae. *PeerJ*, *12*, 1–27. https://doi.org/10.7717/peerj.6089

Valterová, I., Martinet, B., Michez, D., Rasmont, P., & Brasero, N. (2019). Sexual attraction: a review of bumblebee male pheromones. *Zeitschrift Für Naturforschung C*, *74*(9–10), 233–250. https://doi.org/10.1515/znc-2019-0003

Velthuis, H. H. W. W., & van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, *37*(4), 421–451. https://doi.org/10.1051/apido:2006019

Vergara, C. H., & Fonseca-Buendía, P. (2012). Pollination of greenhouse tomatoes by the Mexican bumblebee *Bombus ephippiatus* (Hymenoptera: Apidae). *Journal of Pollination Ecology*, *7*, 285–291. Retrieved from http://dx.doi.org/10.26786/1920-7603%282012%291

Vogt, F. D., Heinrich, B., Dabolt, T. O., & McBath, H. L. (1994). Ovary development and colony founding in subarctic and temperature-zone bumblebee queens. *Canadian Journal of Zoology*, *72*(9), 1551–1556. https://doi.org/10.1139/z94-206

Vogt, F. D., Heinrich, B., & Plowright, C. (1998). Ovary development in bumble bee queens: The influence of abdominal temperature and food availability. *Canadian Journal of Zoology*, *76*(11), 2026–2030. https://doi.org/10.1139/z98-146

Whittington, R., Winston, M. L., Tucker, C., & Parachnowitsch, A. L. (2004). Plant-species identity of pollen collected by bumblebees placed in greenhouses for tomato pollination. *Canadian Journal of Plant Science*, *84*(2), 599–602. https://doi.org/10.4141/P02-192

Williams, P. H., Thorp, R. W., Richardson, L. L., & Colla, S. R. (2014). *Bumble Bees of North America*. Retrieved from http://www.jstor.org/stable/j.ctt6wpzr9

Zhang, H., Huang, J., Williams, P. H., Vaissière, B. E., Zhou, Z., Gai, Q., … An, J. (2015). Managed bumblebees outperform honeybees in increasing peach fruit set in China: Different limiting processes with different pollinators. *PLoS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0121143

CHAPTER 4: CONCLUSIONS

Bumble bees are a charismatic group of bees valued for their role in our food security and ecosystem services (James & Pitts-Singer, 2008; Pitts-Singer et al., 2002; Strange, 2015). Although bumble bees and many other native bees are being studied more than ever, there are still basic components of their natural history that are not well understood. This thesis investigated some of those basic questions that can contribute to supporting bumble bee populations both for ecological restoration and in production for greenhouse pollination on the west coast of North America.

My thesis investigated the efficacy of artificial arboreal nest boxes for attracting bumble bee foundresses and the development of adult male bumble bees as they age. Although these two studies focus on different parts of the bumble bee life cycle, they both shed light on topics that are important for the conservation of this important genus of bee: nest-site preference of sub-alpine/alpine bee communities and the paternal portion of the reproductive cycle. The following are some conclusions that add to the understanding of the bumble bee life cycle from these two experiments.

Many field experiments have been conducted that investigate what attracts bumble bees to nest boxes (Barronet al., 2000; Fye & Medler, 1954; Hobbs et al., 1960; Johnson et al., 2019; Lye et al., 2011). These experiments have been done all over temperate regions with varying bumble bee communities. The aspect and the presence of a blue UV entrance on the nest box showed no significant difference in the number of boxes that the bumble bees interacted with (although boxes on south-facing boxes without a blue/UV entrance had the highest number of boxes that had bees interact with them). This experiment showed high interaction with nest boxes by bumble bees with

34% of the nest boxes having some sort of observation of a bumble bee interacting with the nest box and about 16% of all of the boxes contained bumble bee nests that reached the social phase and produced workers. Results from other nest box or domicile projects have highly variable results but the number of boxes with any detectable interaction in them is fairly high (Johnson et al., 2019). Interestingly, the representation of bees that used the ANBs was not representative of the bumble bee community in the area. Only 5 of the 11 documented species were detected, with the most frequent nester not being the most common bumble bees in the survey zone. This suggests that ANBs may be a valuable tool for studying the ecology and behavior of relatively unknown. The high percentage of boxes having mostly bumble bees interact with them may indicate that montane environments with *B. appositus* may be effective areas for installing these arboreal nest boxes. This experiment should be duplicated, but in other alpine locations where *B. appositus* is a component of the bumble bee community. If the boxes are effective elsewhere then they could be implemented for the conservation of bumble bees by providing habitat, monitoring bumble bee colony phenology and health, and it would be a powerful tool for public outreach and engagement on bumble bee life cycles.

My second experiment was the first to quantify the macro-scale changes of the internal reproductive apparatus that occur as male bumble bees mature, specifically in the Vosnesensky bumble bee. The MIRA of *B. vosnesenskii* shows the visible shrinking of the testes surface area during apoptosis of the germ cells as compared to the accessory testes ending in bees at the age of 8 days; all males having opaque testes indicating the presence of mature spermatozoa by the age of 8 days, and a high concentration of trachea covering the scrotal membrane in almost all male bees by the age of 14 days old. Males showing no difference in reproductive development supports the usage of microcolonies to produce males for captive breeding and justifies the use of microcolony males for further research given no difference in reproductive development between the two. Microcolonies provide greater ease in production of males in captivity because they allow for scheduling of male production and easier extraction with so few workers present guarding the nest. Quantification of the internal development of the bees as they age serves as a reference for future research on male bumble bee development and factors that may affect development rates such as hormones, chemicals, or parasites. This also provides critical data for when males show internal signs of maturity for mating within this commercially viable species *B. vosnesenskii*.

Together these two chapters provide insight into processes occurring during vital stages of the bumble bee life cycle, those being nest establishment and reproductive development. Understanding ways to attract bees to nest in structures in the wild and quantifying changes in male morphology can be used to refine knowledge of bumble bees in their applied use in agroecosystems, in how to better conserve or bolster bumble bee communities, and in understanding their natural evolutionary constraints and pressures. Further investigation in knowledge gaps and establishing baseline data of bumble bee life cycles will better inform the conservation and utility of bumble bees.

Literature cited:

Barron, M. C., Wratten, S. D., & Dovovan, B. J. (2000). A four-year investigation into the effiicacy of domiciles for enhancement of bumble bee populations. *Agricultural and Forest Entomology*, *2*, 141–146.

Fye, R. E., & Medler, J. T. (1954). Field domiciles for bumblebees. *Journal of Economic Entomology*, *47*(4), 672–676. https://doi.org/10.1093/jee/47.4.672

Hobbs, G. A., Virostek, J. F., & Nummi, W. O. (1960). Establishment of *Bombus* spp. (Hymenoptera: Apidae) in Artificial Domiciles in Southern Alberta. *The Canadian Entomologist*, *92*(11), 868–872. https://doi.org/10.4039/Ent92868-11

James, R. R., & Pitts-Singer, T. L. (2008). *Bee pollination in agricultural ecosystems*. Oxford University Press.

Johnson, S. A., Tompkins, M. M., Tompkins, H., & Colla, S. R. (2019). Artificial Domicile Use by Bumble Bees (*Bombus*; Hymenoptera: Apidae) in Ontario, Canada. *Journal of Insect Science*, *19*(7), 1–5. https://doi.org/10.1093/jisesa/iey139

Lye, G. C., Park, K. J., Holland, J. M., & Goulson, D. (2011). Assessing the efficacy of artificial domiciles for bumblebees. *Journal for Nature Conservation*, *19*(3), 154–160. https://doi.org/10.1016/j.jnc.2010.11.001

Pitts-Singer, T. L., Hanula, J. L., & Walker, J. L. (2002). Insect Pollinators of Three Rare Plants in a Florida Longleaf Pine Forest. *Florida Entomologist*, *85*(2), 308–316. https://doi.org/10.1653/0015-4040(2002)085[0308:IPOTRP]2.0.CO;2

Strange, J. P. (2015). *Bombus huntii*, *Bombus impatiens*, and *Bombus vosnesenskii* (Hymenoptera: Apidae) Pollinate Greenhouse-Grown Tomatoes in Western North America. *Journal of Economic Entomology*, *108*(3), 873–879. https://doi.org/10.1093/jee/tov078

APPENDIX

Table S-1

Records of each specific observation of ANBs

Evidence of bee	Year of Census		
	2016	2017	Entire survey
No *Bombus*	64	70	134
Bee entered box	1	1	2
Pollen present	7	7	14
Wax honey pot present	4	1	5
Brood present	10	6	16
Workers present	16	17	33
Total Observations	102	102	204

Table S-2

Specific categorized data on all evidence of bees in ANBs

		Year of survey		
Species	Evidence of bee	2016	2017	Both Years
none		64	70	134
unknown	Sum of evidence	15	13	28
	Pollen present	5	6	11
	Wax honey pot present	4	1	5
	Brood present	6	6	12
B. appositus	Sum of evidence	14	13	27
	Bee entered box	1	0	1
	Pollen present	0	1	1
	Brood present	3	0	3
	Workers	10	12	22
B. centralis	Sum of evidence	4	1	5
	Pollen present	1	0	1
	Workers	3	1	4
B. huntii	Sum of evidence	2	2	4
	Bee entered box	0	1	1
	Workers	2	1	3
B. mixtus	Sum of evidence	0	3	3
	Workers	0	3	3
	Sum of evidence	3	0	3

B. rufocinctus	Pollen present	1	0	1
	Brood present	1	0	1
	Workers	1	0	1
	Total	102	102	204

Table S-3

Name, Location, Elevation, Entrance color, and Aspect of every ANB

Box ID:	Latitude (N)	Longitude (W)	Elevation (ft.)	Painted entrance (Yes/No)	Aspect (North/South facing slope)
S1-N1	41.875945	111.563564	6556	N	North
S1-N2	41.875945	111.563564	6556	N	North
S1-N3	41.875945	111.563564	6556	N	North
S1-B1	41.875945	111.563564	6556	Y	North
S1-B2	41.875945	111.563564	6556	Y	North
S1-B3	41.875945	111.563564	6556	Y	North
S2-N1	41.88623	111.58665	6996	N	North
S2-N2	41.88623	111.58665	6996	N	North
S2-N3	41.88623	111.58665	6996	N	North
S2-B1	41.88623	111.58665	6996	Y	North
S2-B2	41.88623	111.58665	6996	Y	North
S2-B3	41.88623	111.58665	6996	Y	North
S3-N1	41.88676	111.58935	7051	N	North
S3-N2	41.88676	111.58935	7051	N	North
S3-N3	41.88676	111.58935	7051	N	North
S3-B1	41.88676	111.58935	7051	Y	North
S3-B2	41.88676	111.58935	7051	Y	North
S3-B3	41.88676	111.58935	7051	Y	North
S4-N1	41.8872	111.59193	7091	N	North
S4-N2	41.8872	111.59193	7091	N	North
S4-N3	41.8872	111.59193	7091	N	North
S4-B1	41.8872	111.59193	7091	Y	North
S4-B2	41.8872	111.59193	7091	Y	North
S4-B3	41.8872	111.59193	7091	Y	North
S5-N1	41.87337	111.57931	6615	N	North
S5-N2	41.87337	111.57931	6615	N	North
S5-N3	41.87337	111.57931	6615	N	North
S5-B1	41.87337	111.57931	6615	Y	North
S5-B2	41.87337	111.57931	6615	Y	North
S5-B3	41.87337	111.57931	6615	Y	North

S6-N1	41.87739	111.58066	6605	N	South
S6-N2	41.87739	111.58066	6605	N	South
S6-N3	41.87739	111.58066	6605	N	South
S6-B1	41.87739	111.58066	6605	Y	South
S6-B2	41.87739	111.58066	6605	Y	South
S6-B3	41.87739	111.58066	6605	Y	South
S7-N1	41.88651	111.62289	7864	N	North
S7-N2	41.88651	111.62289	7864	N	North
S7-N3	41.88651	111.62289	7864	N	North
S7-B1	41.88651	111.62289	7864	Y	North
S7-B2	41.88651	111.62289	7864	Y	North
S7-B3	41.88651	111.62289	7864	Y	North
S8-N1	41.88927	111.62102	7964	N	South
S8-N2	41.88927	111.62102	7964	N	South
S8-N3	41.88927	111.62102	7964	N	South
S8-B1	41.88927	111.62102	7964	Y	South
S8-B2	41.88927	111.62102	7964	Y	South
S8-B3	41.88927	111.62102	7964	Y	South
S9-N1	41.88512	111.61695	7839	N	North
S9-N2	41.88512	111.61695	7839	N	North
S9-N3	41.88512	111.61695	7839	N	North
S9-B1	41.88512	111.61695	7839	Y	North
S9-B2	41.88512	111.61695	7839	Y	North
S9-B3	41.88512	111.61695	7839	Y	North
S10-N1	41.888	111.61649	7774	N	South
S10-N2	41.888	111.61649	7774	N	South
S10-N3	41.888	111.61649	7774	N	South
S10-B1	41.888	111.61649	7774	Y	South
S10-B2	41.888	111.61649	7774	Y	South
S10-B3	41.888	111.61649	7774	Y	South
S11-N1	41.89144	111.62801	7962	N	South
S11-N2	41.89144	111.62801	7962	N	South
S11-N3	41.89144	111.62801	7962	N	South
S11-B1	41.89144	111.62801	7962	Y	South
S11-B2	41.89144	111.62801	7962	Y	South
S11-B3	41.89144	111.62801	7962	Y	South
S12-N1	41.88894	111.62972	7945	N	North
S12-N2	41.88894	111.62972	7945	N	North
S12-N3	41.88894	111.62972	7945	N	North
S12-B1	41.88894	111.62972	7945	Y	North
S12-B2	41.88894	111.62972	7945	Y	North
S12-B3	41.88894	111.62972	7945	Y	North
S13-N1	41.89183	111.62986	7974	N	South
S13-N2	41.89183	111.62986	7974	N	South

S13-N3	41.89183	111.62986	7974	N	South
S13-B1	41.89183	111.62986	7974	Y	South
S13-B2	41.89183	111.62986	7974	Y	South
S13-B3	41.89183	111.62986	7974	Y	South
S14-N1	41.89161	111.59645	7314	N	South
S14-N2	41.89161	111.59645	7314	N	South
S14-N3	41.89161	111.59645	7314	N	South
S14-B1	41.89161	111.59645	7314	Y	South
S14-B2	41.89161	111.59645	7314	Y	South
S14-B3	41.89161	111.59645	7314	Y	South
S15-N1	41.89212	111.6078	7614	N	North
S15-N2	41.89212	111.6078	7614	N	North
S15-N3	41.89212	111.6078	7614	N	North
S15-B1	41.89212	111.6078	7614	Y	North
S15-B2	41.89212	111.6078	7614	Y	North
S15-B3	41.89212	111.6078	7614	Y	North
S16-N1	41.89725	111.60231	7649	N	South
S16-N2	41.89725	111.60231	7649	N	South
S16-N3	41.89725	111.60231	7649	N	South
S16-B1	41.89725	111.60231	7649	Y	South
S16-B2	41.89725	111.60231	7649	Y	South
S16-B3	41.89725	111.60231	7649	Y	South
S17-N1	41.8964	111.6004	7711	N	South
S17-N2	41.8964	111.6004	7711	N	South
S17-N3	41.8964	111.6004	7711	N	South
S17-B1	41.8964	111.6004	7711	Y	South
S17-B2	41.8964	111.6004	7711	Y	South
S17-B3	41.8964	111.6004	7711	Y	South