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Feeding and Foraging in Bumble bees (Genus: *Bombus*): From the Organism to the Environment

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

Kaleigh Fisher

June 2021

Dissertation Committee:
Dr. S. Hollis Woodard, Chairperson
Dr. Jessica Purcell
Dr. Naoki Yamanaka
Dr. Anupama Dahanukar

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The Dissertation of Kaleigh Fisher is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Feeding and Foraging in Bumble bees (Genus: *Bombus*): From the Organism to the Environment

by

Kaleigh Fisher

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, June 2021
Dr. S. Hollis Woodard, Chairperson

Animal survival is dependent on the capacity to effectively find and consume nutritious food resources and avoid harmful components that may be present in food. This fundamental process operates at multiple scales in all animal species. The goal of this dissertation research was to establish a foundation for studying feeding and foraging in bumble bees at the scales of the organism, colony, insect-plant interactions, and the environment. Although bumble bee feeding and foraging behavior have been previously studied at most of these scales, there are several substantial gaps in our knowledge that this dissertation addresses. First, I first identified taste-related genes in *Bombus impatiens* and characterized the tissues in which these genes are expressed. I then examined how feeding and food-collection tasks are organized amongst workers in bumble bee colonies. Next, I tested whether pollen nutrients impact floral resource visitation in wild bumble bees. Finally, I examined what bumble bee species are present across several ecoregions

in California. Together, this work provides a foundation to study the ecology and evolution of feeding and foraging in bumble bees.

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Introduction

Animal survival is dependent on the capacity to effectively find and consume nutritious food resources and avoid harmful components that may be present in food. This fundamental process operates at multiple scales in all animal species. At the organismal scale, for example, nutritional state impacts whether an individual is motivated to consume or reject food (Dethier, 1976). This motivation is regulated by internal nutritional signaling pathways (Erion & Seghal, 2013). At the scale of animal-food interactions, sensory perception by organisms of visual and chemical cues in food mediates finding and consuming food resources (Stevens, 2013). Finally, community and landscape scale interactions impact the quality and quantity of food available, as well as competitors and predators at food resources (Woodard & Jha, 2017).

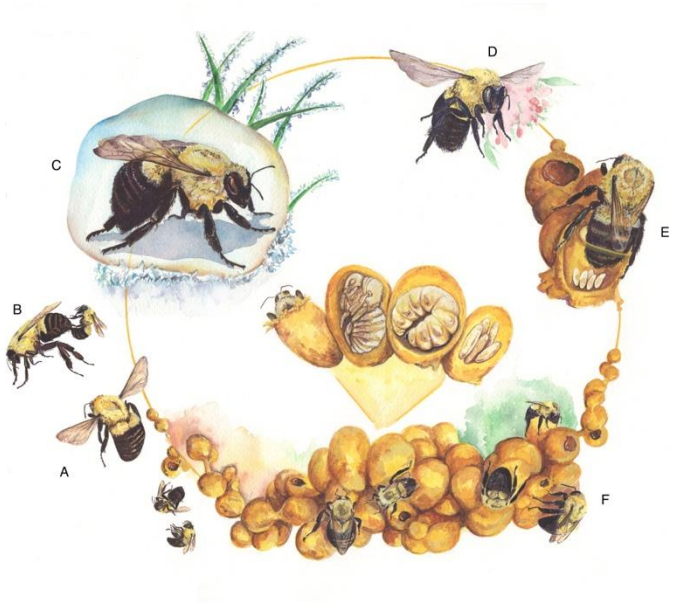

Figure 1: Bumble bee life cycle

The processes of finding and consuming food become more complex in social organisms, where adults may directly feed offspring, and food collection is often coordinated amongst multiple individuals (Fischer & O'Connell, 2017). Bumble bees (Genus: *Bombus*) are a particularly interesting group for studying feeding and food collection because they are social and obtain food resources from a wide range of floral resource species (Michener, 1969). They also directly feed offspring, who have different

nutritional requirements and sensitivities than their own (Heinrich, 2004). As a result, they must discriminate between multiple food choices in their environment and coordinate collection and feeding between multiple individuals.

Bumble bees are a group of annually eusocial bees that consist of ~250 species found globally. They are distributed through North and South America, Eurasia, and parts of Northern Africa (Cameron et al., 2007). Bumble bees are among the most ecologically and economically important pollinators because they are globally widespread and are generalist pollinators, whereby they visit a wide breadth of plant species that include both crop and non-crop plants (Velthuis & van Doorn, 2006). Bumble bee life history is also unique relative to other closely related social lineages, the stingless bees (Genus: *Melipona*) and the honey bees (Genus: *Apis*), because they have both a solitary and social phase of their life cycle whereas both stingless bee and honey bees are perennially social (Michener, 1969). After overwintering (Fig. 1C), a solitary queen emerges in the spring and begins to consume pollen and nectar from floral resources (Fig. 1D) and finds a place to initiate her nest. At this phase in the colony cycle, the queen exclusively feeds for herself. The queen then lays eggs (Fig. 1E). She collects pollen and nectar and stores these resources in the nest until she feeds them to hatched larvae. At this point, the queen forages for floral resources to provision her offspring, as well as to consume herself. When the first offspring emerge as adults (Fig. 1F), they assume the foraging and feeding tasks while the queen transitions to mostly egg laying. Bumble bee workers are much shorter-lived, non-reproductive females. Queens will eventually transition from laying worker offspring to reproductive male and female (gynes) offspring. At the end of the

colony cycle, gynes and males leave the nest (Fig. 1A) and reproduce (Fig. 1B). The mated gynes are the only individuals that enter diapause and survive over winter (Fig. 1C), after which the cycle continues (Sladen, 1912). Because the bumble bee life cycle has a solitary and social phase, it is a tractable system for disentangling the complexity of feeding in social insects. For example, it is possible to explore both individual feeding behavior and social feeding behavior (brood feeding through maternal or sibling care) within the same species.

The goal of this dissertation research was to establish a foundation for studying feeding and foraging in bumble bees at the scales of the organism, colony, insect-plant interactions, and the environment. Although bumble bee feeding and foraging behavior have been previously studied at most of these scales, there are several substantial gaps in our knowledge that this dissertation addresses. First, the primary tissues that are used by bumble bees to facilitate taste, a sensory modality involved in feeding and foraging behavior (Hallem et al., 2006), are currently unknown in bumble bees. Tissues where gustatory receptor (GR) genes are expressed, which encode gustatory receptors on gustatory receptor neurons (GRNs), provide evidence that those tissues are involved in taste (Scott et al., 2001). Moreover, it is unknown whether these genes are differentially expressed between the two castes that perform feeding and foraging behavior, the queens and workers. Differential expression of genes between these two castes would suggest that these genes are involved in different behavioral processes, potentially related to feeding. To begin to address this gap, in **Chapter One** I identified taste-related receptor genes in the bumble bee *B. impatiens*, and compared gene duplication and loss between

this species and *B. terrestris*, *M. quadrifasciata*, and *A. mellifera,* species where they have previously been identified (Sadd et al., 2016; Brand & Ramirez, 2017). I then compared differences in gene expression of the two subfamilies of taste related genes: the gustatory receptors (GRs) and ionotropic receptors (IRs) (Chen & Dahanukar, 2019). Specifically, I compared gene expression of these genes in three peripheral tissues (mouthparts, antennae, and tarsi) and two internal tissues (brain and fat body) in the common eastern bumble bee *B. impatiens*. I then compared gene expression in these tissues between the queen and worker caste. Identifying where taste-related genes are expressed is the first necessary step to perform in order to ultimately characterize the specific functional role of these genes (i.e., what compounds they can detect) and how they impact feeding and foraging behavior (Clyne et al., 2000; Scott et al., 2001). Moreover, identifying caste-specific expression patterns in any of these genes will help to illustrate their potential functional roles.

The second gap addressed in my dissertation was resolving how feeding and food collection is coordinated between individuals in the bumble bee colony. In **Chapter Two**, I observed brood feeding and food collection behavior in early-stage nests of *B. impatiens*. I directly observed these behaviors in nests with four different social configurations to see if the number of workers and the presence of the queen impact the coordination of these behaviors: three workers and a queen, three workers without a queen, five workers with a queen, and five workers without a queen. Only one study to date has directly observed both feeding behavior and food collection in individual bumble bees, at any colony stage (Brian, 1952). Describing which individuals directly feed brood

and collect food is important for future studies that can examine how these behaviors are nutritionally regulated at the colony level. More fundamentally, this foundational work helps inform whether the individuals that perform these behaviors have differences in taste sensory capacity.

Next, in **Chapter Three**, I explored the role of pollen nutrients in mediating floral resource visitation by wild bumble bee workers. We are currently unable to predict what plants bumble bee will visit in a natural landscape; the presence of specific pollen nutrients may be a mechanism that explains floral visitation (Woodard & Jha, 2017). Identifying the nutrients that bumble bees collect in pollen is an important step in order to perform functional studies on what compounds taste-related receptors are activated by. Candidate nutrients are thus essential to generate a taste panel that can be used for further experiments to describe what chemical compounds in food resources bees can detect and discriminate between. I performed metabolomic analyses on bumble bee collected pollen from two species and in different ecoregions throughout the Sierra Nevada Mountains. Results from this chapter provided evidence as to whether pollen nutrients impact floral resource visitation, and if different species collect different nutrients in pollen.

Finally, in **Chapter Four** I surveyed the distribution of bumble bees throughout California in order to identify the bumble bee assemblage composition in different ecoregions, which are assigned based on habitat characteristics (Griffith et al., 2016). I collected ~100 bees from 17 sites, distributed across six ecoregions in California. Results from this chapter provide an important update on the status and distribution of the bumble bee assemblage throughout the state of California. Species surveys shed light on what

types of habitats support different species and can elucidate potential competitive interactions between species that can ultimately impact foraging behavior in bumble bees. There has not been a statewide survey of bumble bees in California since 1983 (Thorp, 1983); thus this survey was particularly timely.

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Chapter One: Tissue- and caste-related expression of gustatory receptor genes in the bumble bee *Bombus impatiens*

Abstract

Taste is a core animal sensory system that is essential for survival. Insects use vision and smell to find food resources, but it is taste that ultimately regulates feeding behavior by operating as a first line of defense for differentiating between beneficial (e.g., nutritive) and harmful (e.g., toxic) compounds in food. Taste is mediated by gustatory receptors housed in gustatory receptor neurons, which are encoded by gustatory receptor genes. In order to build a foundation to study taste in bumble bees, I annotated gustatory receptor genes in the common eastern bumble bee, *Bombus impatiens* and compared patterns of duplication and loss with *B. terrestris*, *Melipona quadrifasciata*, and *Apis mellifera*. I then compared gene expression of these genes in the major taste tissues, and between the queen and worker castes. I found that *B. impatiens* have three GR expansions, similar to *B. terrestris* and that GRs are primarily expressed in the mouthparts and antennae.

Introduction

Taste is a core animal sensory system that is essential for survival (Hallem et al., 2006). Insects use vision and smell to find food resources, but it is taste that ultimately regulates feeding behavior by operating as a first line of defense for differentiating between beneficial (e.g., nutritive) and harmful (e.g., toxic) compounds in food (Chittka & Raine, 2006; Kessler et al., 2015; Muth et al., 2016). Taste is also important for other fundamental behaviors, such as mating (Watanabe et al., 2011) and selection of oviposition sites in insects (Scott, 2018). Upon contact with detectable compounds in the environment (ligands), taste receptors transmit a signal to the brain, where peripheral taste information is integrated with additional, internal information; this can ultimately result in either acceptance or avoidance behavior (Hallem et al., 2006). In addition to the taste system underlying key behaviors in insects, differences in sensory tuning of taste receptors have been associated with differences in resource use between closely related lineages (Diaz et al., 2018; McBride et al., 2007a). Thus, this sensory system may be a key driver of insect diversification. Despite the critical importance of taste to insect behavior, ecology, and evolution, much remains to be understood about how it operates in different insect lineages (Robertson, 2019).

Taste is mediated by taste-related receptors located in gustatory receptor neurons (GRNs). Most taste receptors in GRNs are gustatory receptors (GRs), which are encoded by the gustatory receptor gene family (Freeman & Dahanukar, 2015). Since the discovery of GR genes in *Drosophila melanogaster,* the first taste-related genes to be found in any insect (Clyne et al., 2000), significant insights have been made towards understanding

and characterizing how taste operates in flies and several other insects (Scott, 2018). For example, it is now known that insect taste organs include the mouthparts, tarsi, wing margins, and ovipositor (Scott et al., 2001). The identification of taste-related genes, and the organs in which they are expressed, has been essential to developing targeted assays to uncover the ligands that taste receptors are tuned to. This information is ultimately critical for elucidating the breadth of compounds insects can detect in their environment, and how this impacts insect behavior and survival.

GR genes are highly conserved throughout the insects (Kent & Robertson, 2009; Robertson, 2019). They have been annotated in a number of insect species, including in several *Drosophila* species (Clyne et al., 2000; McBride et al., 2007b), several disease vectors and pest species (Obiero et al., 2014; Mesquita et al., 205; Benoit et al., 2016; Robertson et al., 2019), insect species with more ancestral traits like damselflies (Ioannidis et al., 2017), and several social insects (Robertson & Wanner, 2006; Smith et al., 2011a; Smith et al., 2011b). The GR gene family has evolved in insects through birth-death evolution, which includes divergence, duplication, loss, and pseudogenization (Nei et al., 2008). The family includes conserved sugar and bitter sensing subfamilies, as well as a conserved fructose receptor lineage (Robertson, 2019). In addition to GRs, some members of the ionotropic receptor (IR) gene family have also been shown to play a role in the taste system in *D. melanogaster* (Benton et al., 2009; Croset et al., 2010), specifically in the detection of amino acids (Chen & Dahanukar, 2019; Ganguly et al., 2017). Additional components of the taste system include genes in the pickpocket (Cameron et al., 2010) and transient receptor potential (Kang et al., 2012) families. The

discovery of taste-related genes in more insect species has helped to shed light on how these gene families evolved across the insect phylum (Kent & Robertson, 2009). This has also enabled functional studies on how taste operates in different lineages (Scott, 2018; Lim et al., 2019; Chen & Dahanukar, 2019). Comparative genomic studies have additionally shown that there is significant variation in the number and diversity of taste receptors across lineages (Robertson, 2019). Across organisms, there is broad support that diversification and expansion of closely related genes often gives rise to changes in gene function and ecological adaptations (Mcbride, 2007a; Diaz et al., 2018).

Here I examined the evolution and expression of taste-related genes in the common eastern bumble bee, *B. impatiens*. This is an important first step in understanding how taste works in systems, such as bumble bees, where our knowledge of taste is still limited (Clyne et al., 2000) because it established a starting point for future studies to examine how this process relates to a species' behavior and ecology. Bumble bees have undergone three recent GR gene expansions (Sadd et al., 2015) that are not shared with the closely related honey bee (Robertson & Wanner, 2006), and the functional significance of which is currently entirely unknown. *B. impatiens* is one of the most commonly used bumble bees for commercial pollination (Velthuis & van Doorn, 2006) and also in both laboratory and field studies. More broadly, bumble bees are important native pollinators globally, and are an emerging model system for understanding the molecular basis of behavior, ecology, and evolution (Woodard et al., 2015). First, we assessed evolutionary relationships between GRs and IRs in *B. impatiens* and a set of additional genomes in the clade to which this species belongs. This analysis

was necessary to establish whether specific GR and IR genes are lineage-specific or are present in the broader group of bees. Next, to begin to understand how taste functions in *B. impatiens*, we generated a spatial map of GR and IR receptor expression. For this, we assessed the queen and worker castes separately to explore whether there are caste-specific differences and focused on tissues where GRs are known to be expressed in other insects. Identifying spatial patterns of taste-related receptor expression is an important step towards uncovering the function of different taste genes. For example, this information is essential for developing and implementing targeted behavioral assays that can help elucidate receptor functions (Scott et al., 2001).

Methods

Gene annotations and phylogenetic comparisons

I annotated GR and IR protein sequences for *B. impatiens* from the BIMP 2.0 genome using the same methods previously used to annotate chemosensory genes in *B. terrestris* (Sadd et al., 2015), *A. mellifera* (Robertson & Wanner, 2006), and *M. quadrifasciata* (Brand & Ramirez, 2017). We obtained GR and IR protein sequences used in previous studies of these additional bee species, in order to identify orthologs with *B. impatiens*. *B. impatiens* and *B. terrestris* belong to two different subgenera, Pyrobombus and Bombus, respectively, in the *Bombus* genus (tribe Bombini; Cameron et al., 2007). *M. quadrifasciata* belongs to the sister group to Bombini (the Meliponini), and *Apis* is the outgroup to this clade (Apini). *Bombus*, *Melipona*, and *Apis* are all eusocial, although *Bombus* has both a solitary and a social phase of an annual nesting cycle, whereas

Melipona and *Apis* have perennially nests and no solitary phase (Michener, 1969). To compare GR and IR evolutionary patterns between these closely related lineages, we aligned all sequences for GRs and IRs, respectively, for all species using ClustalOmega (McWilliam et al., 2013) and manually inspected all alignments to ensure that they represented consistent sequences between species. We then generated distance-based neighbor joining trees, using the R packages Phanghorn and Ape, as input for maximum likelihood models to find the best tree model. We used the *omptim.pml()* function, with stochastic substitutions, to find the best fitting trees. Trees were rooted at the sugar receptors, GR1 and GR2 (Robertson, 2019), for gustatory receptors, and IR8 and IR25a for ionotropic receptors (Croset et al., 2010). Both of these groups are highly conserved throughout the insects and likely the basal groups for both chemoreceptor lineages (Croset et al., 2010).

Bumble bee rearing

Three queen-producing and three worker-producing *B. impatiens* colonies were supplied by Koppert Biological Systems (Howell, MI). Twenty newly-eclosed (age < 24 h, or "callow") individuals were removed from each colony (queen: n = 3; worker: n = 3) and placed into rearing cages, supplied by Biobest USA, Inc. (Romulus, MI, USA) in colony groups. Callow individuals were identified by their silvery appearance. Seven days after eclosion, bees were frozen at -80°C and stored at this temperature until tissue dissections. These methods allowed us to collect individuals that were age-matched (at adult age seven days) and collected at the same time of day to control for age- and circadian-related differences in gene expression. All colonies and bees were maintained

in either Biobest queen cages, or Koppert colony boxes, in groups of at least five bees. All bees were maintained under standard rearing conditions (25 ± 3°C, 40 ± 5% RH) and supplied with honey bee-collected pollen (stored frozen; obtained from Biobest USA, Inc. Romulus, MI) and artificial nectar syrup (Boyle et al., 2018), both provided *ad libitum*.

Sample preparation, RNA isolation and sequencing

The following tissues were dissected over dry ice: proboscises, which included the glossa, labial palps, and maxillary palps; antennae; front tarsi; brain; and fat body. Proboscises were cut at the base of the clypeus. Both antennae were removed at the base of the head capsule and combined, where a total of 40 antennae from 20 individuals were pooled. Front tarsi were removed from the tibia and combined. Brains were removed from the head capsule through the dorsal side and the subesophageal ganglion was removed. Fat bodies were obtained from abdomens and were left connected to the abdominal cuticle (Costa et al., 2020). For each tissue type, tissues from twenty individuals from the same source colony were pooled and stored in Trizol at -80°C until RNA extraction. Tissues were homogenized in Trizol with two metal beads at maximum frequency for 5 min using a TissueLyser II (Qiagen). RNA was isolated from homogenized tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Per sample RNA quality and quantity were assessed using agarose gel electrophoresis, nanodrop and an Agilent 2100 bioanalyzer. RNA sequencing libraries were generated from 800 ng of total RNA per sample using Illumina's TruSeq Stranded mRNA Sample Prep Kit following the manufacturer's instructions. RNA libraries were

multiplexed and sequenced with 150-bp paired-end reads (PE150) to a depth of ~20 million reads per sample on an Illumina HiSeq 4,000 at the Novogene Corporation Labs at UC Davis.

Differential gene expression analysis

We used the package *Deseq2* (Love et al., 2014) to identify differentially expressed genes. We used median of ratios to generate normalization factors of gene expression for each sample, to account for differences in sequencing depth and RNA composition between samples, using a generalized linear model (glm), with caste, tissue, and the interaction between caste and tissue, as factors. Genes with zero counts across all samples, extreme outliers, and low mean normalized counts were filtered from the dataset. Normalized counts of the complete dataset were then regularized log (*rlog*) transformed to moderate the variance across the mean to visualize samples for quality control. We assessed sample quality and evaluated whether variance between samples could be explained by any of the factors in the glm, or natal colony, using the top 500 most variable genes in the dataset, in order to inform which factors should be included in the final generalized linear model. For this we used a principal component analysis (PCA) and hierarchical clustering analysis. Based on our quality control analysis, which demonstrated that variance between samples could be explained by caste and tissue, but not natal colony, we tested the effect of caste, tissue, and the interaction between caste and tissue on gene expression using a glm with a negative binomial distribution.

***Comparisons of chemoreceptor expression patterns across* samples:** We performed Wald tests using every gene in the complete dataset, with a Benjamini-Hochberg FDR

correction ($p < 0.05$) to account for multiple comparisons. Results were then filtered to only include annotated gustatory and ionotropic receptors, to test whether chemoreceptor gene expression differed between tissues and castes. Normalized counts of chemoreceptor genes were then used to visualize expression differences between genes and to visualize similarity in chemoreceptor expression between tissues and castes using a non-metric multidimensional scaling analysis (NMDS) with a Bray-Curtis dissimilarity index. This was followed by an analysis of similarity (ANOSIM) in the Vegan package (v. 2.5-6; Oksanen *et al*., 2019) to test whether there were statistical differences in gene expression between tissues and castes.

Results

Evolutionary analysis of gustatory receptors

We identified 18 intact GR genes in *B. impatiens* and three pseudogenes (Table 1.1). The intact genes include two putative sugar receptors, one putative fructose receptor, and 15 putative bitter receptors, based on orthology to *D. melanogaster* (Robertson et al., 2003) and *A. mellifera* (Robertson & Wanner, 2006). Of the 15 putative bitter receptors, 9 are part of the three gene family expansions: four are in the GR8 family expansion, one is in the GR9 family expansion, and four are in the GR12 family expansion (Fig. 1.1). *B. impatiens*, *B. terrestris*, *M. quadrifasciata*, and *A. mellifera* all share the two putative sweet GRs and the putative fructose GR. Each of these bee species also has several putatively bitter GR genes, with the most being annotated in *B. terrestris*. *B. impatiens* only has two intact genes in the GR9 family expansion, one pseudogene,

and one gene split between two scaffolds compared to four intact genes in *B. terrestris*. *B. terrestris* also has one more gene in the GR12 family expansion compared to *B. impatiens*. *M. quadrifasciata* has thirteen GR genes (Brand & Ramirez, 2017), with three intact GRs and one pseudogene in the GR12 family expansion, whereas *A. mellifera* only has a single gene copy (Robertson & Wanner, 2006).

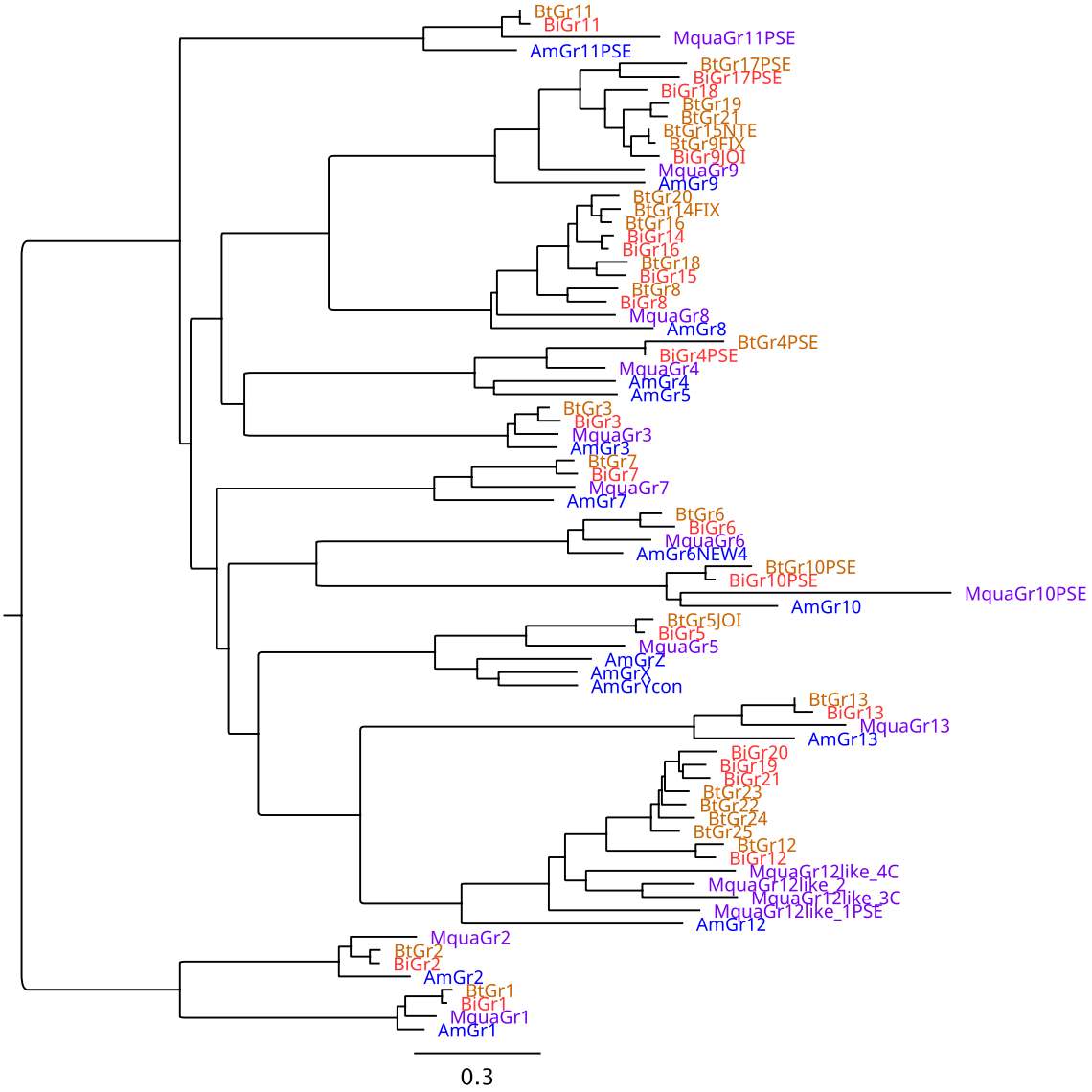

Figure 1.1: *Gustatory receptor gene tree. Tree was constructed using the neighbor joining method. The tree is rooted at Gr1 and Gr2, which are highly conserved across insects, which suggests they are the more basal gene group.*

Evolutionary analysis of ionotropic receptors

I found 22 intact IR genes in *B. impatiens* and no pseudogenes (Table 1.1). All intact IR genes are 1:1 orthologs with *B. terrestris*. Four of these genes are 1:1 orthologs with *D. melanogaster* genes (Sadd et al., 2015), all of which are highly conserved across the insects (Croset et al., 2010). There are two IR subfamilies of IRs in insects: 1) The antennal IRs are highly conserved throughout the insects and include IR8a, IR25a, IR68a, IR75u, IR76b, and IR93a, whereas; 2) the species-specific divergent IRs have previously been demonstrated to be expressed in gustatory receptor neurons in other insects, including the *A. mellifera* (Croset et al., 2010). We identified 22 IR genes in *B. impatiens*, the same number and copies found in *B. terrestris*. Seven of these include the antennal IRs; IR25a has two gene copies and are referred to as IR25a.1 and IR25a.2 in *B. terrestris* (Sadd et al., 2015), and hereafter in *B. impatiens*. However, IR25a.2 only encodes the second half of the protein, but is otherwise a functional gene (Sadd et al., 2015). The remaining 17 genes are orthologous to *A. mellifera*, in addition to *B. terrestris*, with the exception of IR333, which is a paralog in *Bombus* to IR332 and not present in *A. mellifera* (Sadd et al., 2015). These genes are highly divergent from *D. melanogaster*, the organism where IR functionality has been almost exclusively characterized (Figure 1.2; Sadd et al., 2015). *M. quadrifasciata* shares the six antennal IR orthologs, but only four of the divergent genes that are orthologous to *A. mellifera* and *Bombus* (Figure 1.2). *Bombus* is sister group to *Melipona* and *Apis* is an outgroup.

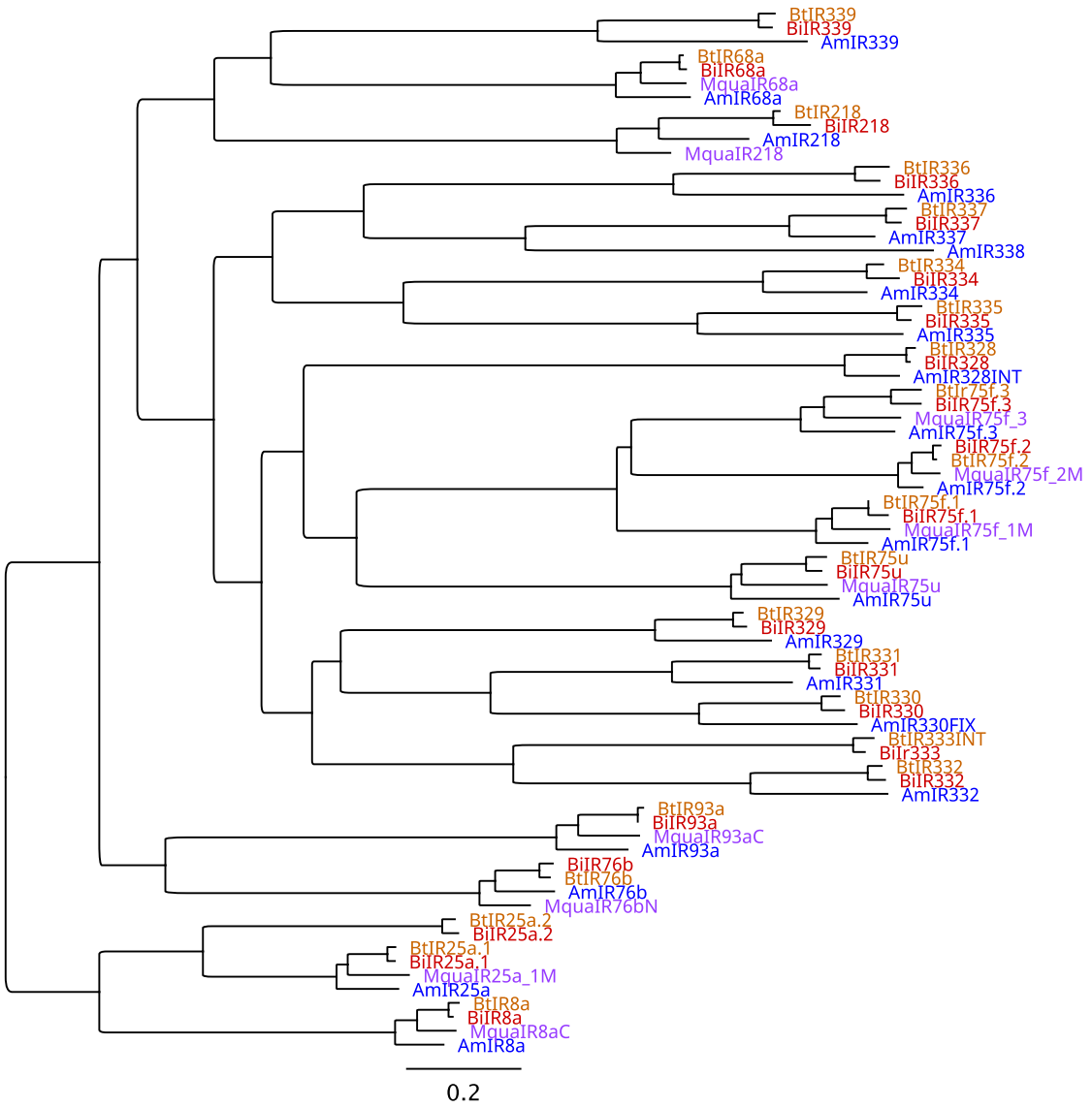

***Figure 1.2**: Ionotropic receptor gene tree. Tree was constructed using the neighbor joining method. The tree is rooted at IR8a and IR25a, which are highly conserved across insects, which suggests they are the more basal gene group.*

Species	GRs	GR Pseudogenes	IRs	IR Pseudogenes
Bombus impatiens	18	3	22	0
Bombus terrestris	21	3	22	0
Melipona quadrifasciata	13	3	10	0
Apis mellifera	12	1	21	0
Drosophila melanogaster	60	2	64	4

Table 1.1: Numbers of GR and IR genes in *B. impatiens* and other closely related species **Drosophila melanogaster* genes from Scott et al., 2001; Benton et al., 2009; Robertson, 2019; *Apis mellifera* genes from Robertson, 2006; *Melipona quadrifasciata* genes from Brand & RamIRez, 2017; *Bombus terrestris* genes from Sadd et al., 2015.

Transcriptome analysis

Our transcriptome dataset included 14,860 *B. impatiens* gene transcripts, representing 93.5% of all coding genes in the genome (Sadd et al., 2015). 5,382 (36.22%) of these genes were differentially expressed between samples (FDR-corrected $p < 0.05$). All chemoreceptors identified in the dataset were expressed in most tissues and expressed in both the queen and worker castes.

Gustatory receptor expression

Thirteen of the eighteen intact *B. impatiens* gustatory receptors were identified in the RNAseq dataset. Three genes that were not present, GR18, GR19, and GR21, are a part of the *Bombus*-specific gene family expansions (Fig. 1.1). GR13, which is missing the C-terminus but is otherwise an intact gene (Sadd et al., 2015), and GR9, which is on two separate scaffolds in the genome, were not identified in the transcriptomic dataset.

GR Sugar receptors: Both GR1 and GR2, which are putatively involved in sugar detection, were expressed in all tissues, with the highest expression levels detected in the mouthparts and antennae for GR1 (FDR corrected $p < 0.05$). GR1 expression was also higher in worker versus queen mouthparts (FDR corrected $p < 0.05$). Though GR2 was expressed at relatively high levels in the antennae and mouthparts as well, expression was also high in the tarsi and fat body.

GR Fructose receptors: GR3, which is putatively involved in fructose detection, was expressed at relatively high levels in all tissues, with highest expression in the brain relative to all other tissues (FDR corrected $p < 0.05$). GR3 was also expressed in the queen fat body at higher levels than worker fat body.

GR8 family expansion: GR8, GR14, and GR16, which is putatively involved in bitter detection based on orthology, but whose specific functions are completely unknown, were all expressed at higher levels in the antennae compared to other tissues (FDR corrected $p < 0.05$). All of these genes were expressed in all tissues, with the exception of GR14, which was not detected in the fat body. GR15 expression was higher in the antennae and mouthparts compared to other tissues, and lower in the tarsi compared to the brain (FDR corrected $p < 0.05$). GR8 was also expressed at higher levels in the worker versus queen fat body.

GR9 Family expansion: GR18 expression was higher in the antennae than all other tissues, except mouthparts (FDR corrected $p < 0.05$). There were no differences in expression between castes. RNA expression was not detected in Gr9 or Gr17 because they were not found to be functional genes.

GR12 Family expansion: The Gr12 family expansion is shared with *M. quadrifasciata* but not *A. mellifera* and is likely involved in the detection of bitter compounds, but the role of these receptors in the detection of specific compounds is unknown in all of these species. GR12 expression was higher in the mouthparts than fat body and tarsi, where expression was not detected. GR20 expression was not detected in the fat body or tarsi, and expression was low in some antennal and brain samples, and not detected at all in other antennal and brain samples. There were no differences in the GR12 family expansion gene expression between castes.

Other genes: GR5 was expressed at higher levels in the antennae than brain and fat body, where in the latter two tissues it was not expressed at detectable levels. GR7 was

expressed at higher levels in the antennae and mouthparts compared to the brain, fat body, and tarsi. It was also expressed at a higher level in the tarsi compared to the brain and fat body. GR11 was expressed at the highest level in the brain relative to all other tissues, and expression was higher in tarsi than fat body. There were no differences in expression between castes. The exact compounds that the receptors may be involved in detecting are unknown.

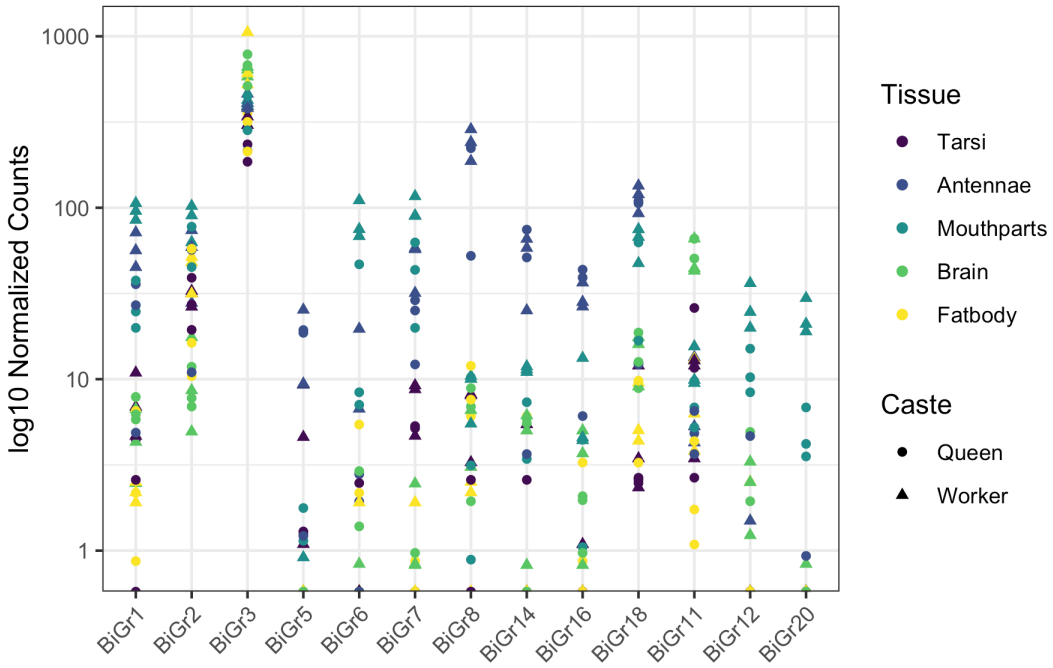

Figure 1.3*: Normalized counts of gustatory receptor gene expression in queens and workers. Points are jittered for easier visualization of overlapping points (width +/- 0.05; height +/- 2).*

Gustatory Receptor Clustering: There were differences in similarity between tissue samples, where internal tissues were more similar based on GR expression, which included the fat body and brain, compared to the external tissues, which included tarsi, mouthparts and antennae (Figure 1.4; $R^2 = 0.981$; stress = 0.13; ANOSIM Tissue Type: $R = 0.39$; $p < 0.001$).

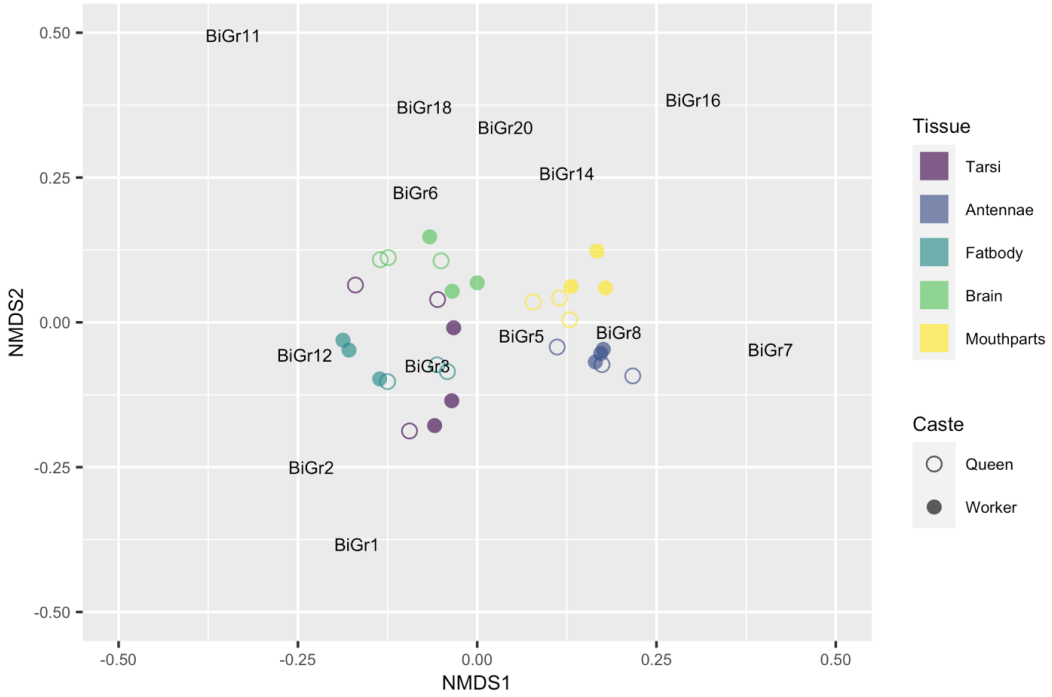

Figure 1.4*: Non-metric multidimensional scaling of samples based on similarity in GR expression of tissue and caste. Distance between points represents similarity in gene expression. Internal tissues were more similar to each other compared to external tissues. Greater similarity within a caste compared to between castes was not observed.*

Ionotropic receptor expression

We identified 20 unique IR genes in the RNAseq dataset out of the 22 annotated IR genes in the genome. IR75f.3 was not present in the dataset. IR25a.1 was not present

in the dataset, likely because it only encodes the second half of the receptor protein and thus may not have been expressed (Sadd et al., 2015).

Antennal IRs: These IRs were originally discovered in the antennae in *D. melanogaster* (Croset et al., 2010), and the function of which are largely unknown in all insect species. IR8a was expressed at higher levels in antennae than all other tissues (FDR corrected $p < 0.05$). It was also expressed at higher levels in the brain relative to mouthparts, fat body, and tarsi. IR25a.2 was expressed at higher levels in the brain than all other tissues (FDR corrected $p < 0.05$). This gene was also expressed at higher levels in the antennae relative to the remaining tissues. IR76b expression was higher in brain than mouthparts, and lower in mouthparts compared to antennae. IR76b expression was also lower in worker fat body relative to brain and mouthparts, but not queen fat body. IR93a expression was highest in mouthparts and antennae, and lowest in fat body and brain (FDR corrected $p < 0.05$). Tarsal expression was higher than brain and lower than antennae, but not statistically different from mouthparts or fat body. IR75u expression was high in all tissues and higher in antennae than all tissues except for tarsi, with the lowest expression in the brain. IR75u fat body expression was higher in workers compared to queens. IR68a expression was highest in fat body for both castes and was higher in the queen fat bodies compared to workers. IR76b was expressed at higher levels in queen tarsi compared to worker tarsi. IR93a, IR68a, and IR75u were expressed at higher levels in worker tarsi compared to queen tarsi.

Bee-specific divergent IRs: These IRs are specific to bee lineages and have completely unknown functions. IR75f.1 was highest in the fat body and lowest in the brain. IR75.2

expression was high in all tissues, and highest in the mouthparts. IR332 was highest in antennae. IR329 was high in all tissues. IR330 and IR331 had the highest expression in fat body and mouthparts. IR328 had highest expression levels in worker fat body. IR339 expression was highest in the antennae. IR218 had higher expression in worker fat body compared to queens. IR337 was expressed in most tissues but had low levels in tarsi. IR336 had highest expression in the mouthparts, and lowest in the fat body. IR335 had low expression levels in most tissues, particularly in the mouthparts. IR334 had highest expression levels in the antennae compared to other tissues. IR328 was expressed at higher levels in queen tarsi compared to worker tarsi. IR218, IR329, IR330, and IR331 were expressed at higher levels in worker tarsi compared to queen.

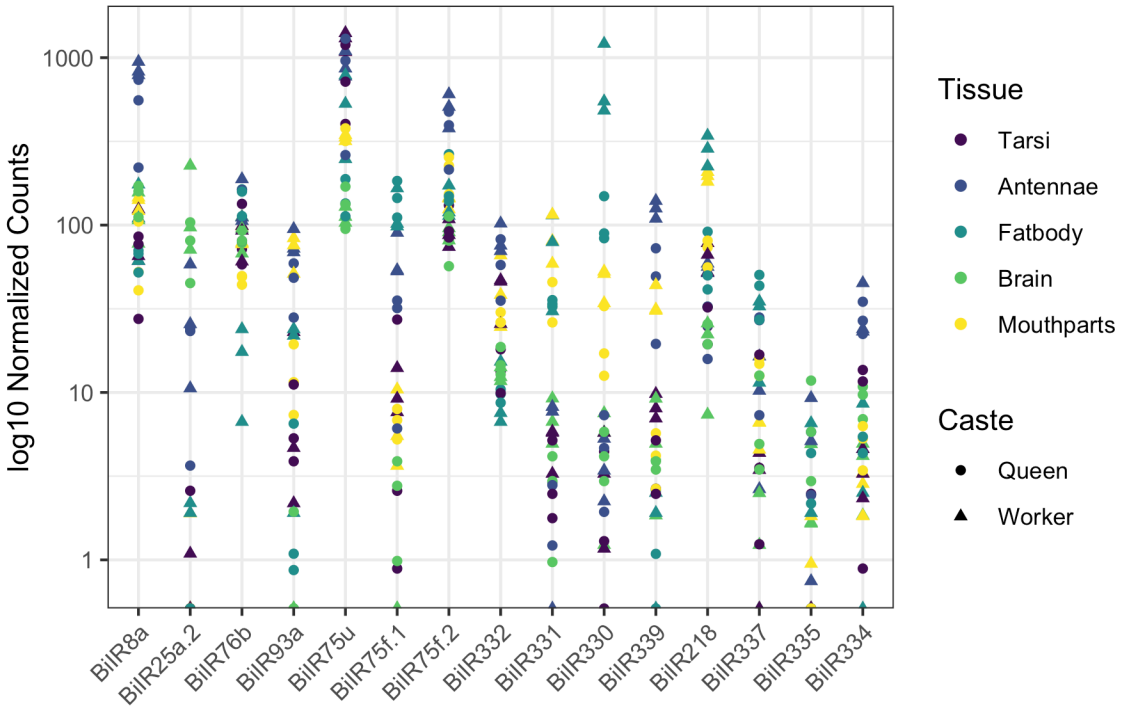

Figure 1.5*: Normalized counts of ionotropic receptor gene expression. Points are jittered for easier visualization of overlapping points (width +/- 0.05; height +/- 2).*

Ionotropic Receptor Clustering*:* There were differences in similarity between tissue samples, where internal tissues were more similar based on GR expression, which included the fat body and brain, compared to the external tissues, which included tarsi, mouthparts and antennae (Figure 1.6; $R^2 = 0.984$; stress = 0.12; ANOSIM Tissue Type: $R = 0.59$; $p < 0.001$). Further, fat body and brain were also distinct (ANOSIM Fat body: $R = 0.66$; $p < 0.001$). There was no difference in similarity between castes (ANOSIM Caste: $R = 0.06$; $p > 0.05$).

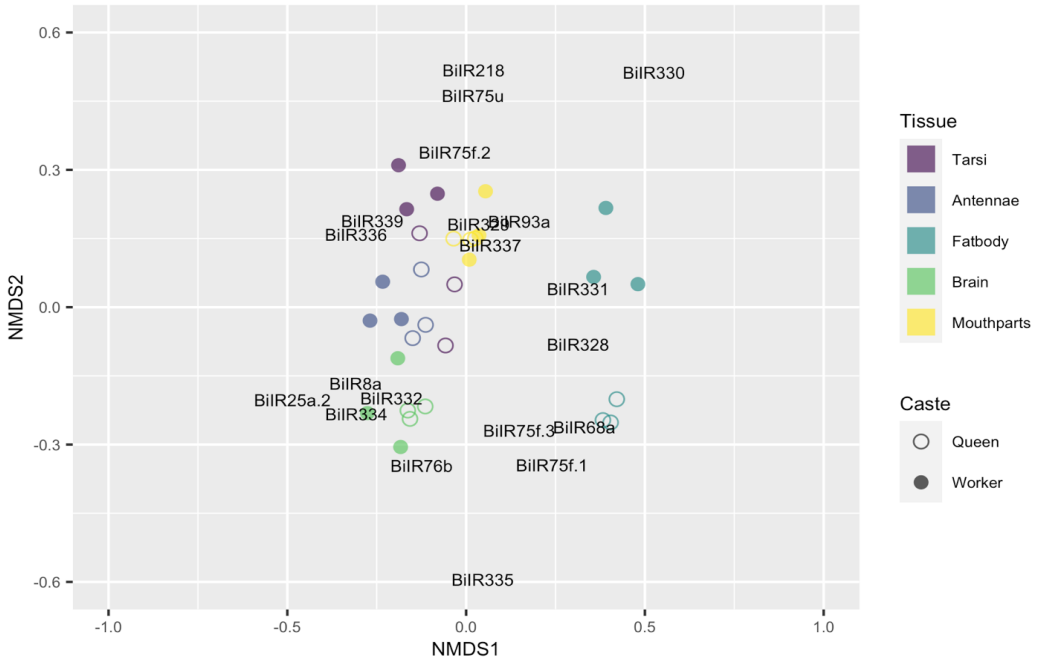

Figure 1.6*: Non-metric multidimensional scaling of samples based on similarity in IR expression of tissue and caste. Distance between points represents similarity in gene expression. Internal tissues were more similar to each other compared to external tissues. Greater similarity within a caste compared to between castes was not observed. Fat body was dissimilar to brain within the internal tissues.*

Discussion

In this study, we explored the evolution, and tissue and caste-related expression patterns, of gustatory and ionotropic receptor genes, in the bumble bee *B. impatiens*. Comparative examinations of GR and IR genes have been performed between the honey bee *A. mellifera* and the bumble bee *B. terrestris* (Sadd et al. 2015), but not in the North American model bumble bee species *B. impatiens,* nor in the stingless bees, the sister group to the bumble bees. There has historically been a focus on the importance of vision and olfaction to bee foraging behavior. Further, the first fully sequenced bee genome, for the honey bee *Apis mellifera*, had a significant reduction in GR genes compared to *D. melanogaster,* and had fewer GR genes compared to olfactory receptor genes. However, the discovery of three GR gene family expansions in the bumble bee *Bombus terrestris* provided genomic evidence that taste may also be an important sensory modality in this bee lineage. The subsequent discovery of IR genes in *D. melanogaster* taste neurons (Benton et al., 2009) and later in other insects (Crosset et al., 2010), including bumble bees (Sadd et al., 2015), provided further evidence that taste may be a more important sensory modality in bees than previously thought.

Consistent with a recent analysis of genes in the *B. terrestris* genome (Sadd et al. 2015), we found that the bumble bee *B. impatiens* also has an expanded set of GR-encoding genes, relative to honey bees. However, there are also species-specific differences within the bumble bee genus (*Bombus*), given that we annotated 18 intact GRs in *B. impatiens*, compared to 21 in *B. terrestris*. Chemoreceptor gene families are among the fastest evolving gene families in the animal kingdom (Nei, 2008) and species-

specific paralogous gene duplicates have been identified in a number of insect lineages. These gene duplicates tend to diverge quickly and are under positive selection (Almeida et al., 2014), and is likely driven by differences in detection of ligands (Robertson, 2019). Although *B. terrestris* and *B. impatiens* are both generalist pollinators, they are exposed to different floral resources and nesting behavior, both of which could be selection pressures that are shaping these different patterns in GR evolution.

The shared expansion of the GR12 gene family, but the reduction in IR genes in *Melipona*, suggests that GRs may have begun to expand with the common ancestor of *Melipona* and *Bombus*, and that the divergent IRs may have duplicated in the common ancestor of the corbiculate bees. Further comparisons with the Euglossini (the corbiculate lineage that is the outgroup to the lineages examined in this study), outgroups of the corbiculate bees, and other, more distantly related bee and Hymenopteran species, will further resolve these patterns of gene duplication and loss. However, we caution that the accuracy of these evolutionary relationships is fully dependent on the quality of genome assembly and annotation. For example, gaps in sequencing of the *B. impatiens* genome may explain the lack of intact genes in the GR9 family expansion and the missing C-terminus in GR13 for example, and further sequencing will elucidate whether these genes are actually functional or pseudogenes.

Identifying the expression patterns of chemoreceptors helps to elucidate their function because describing the tissues where they are present can inform what behaviors they are involved in. This information is also important so that functional studies can be performed to identify which ligands receptors are activated by (Chen & Dahanukar,

2019). Identifying the ligands that receptors are tuned to can in turn contextualize the evolutionary patterns of gene duplication, loss and pseudogenization (Robertson et al., 2019). We detected GR and IR expression in both peripheral and internal tissues, and similarity in expression patterns could be explained by whether the tissues were peripheral or internal. Within the internal tissues, IR expression followed different patterns in the fat body compared to the brain, though they were more similar to the brain than the peripheral tissues. Receptors present in peripheral tissues like the antennae, tarsi, and mouthparts, may be involved in discriminating between compounds in the nest and floral resources, whereas those expressed in internal tissues may be involved in internal regulation of nutrient metabolism (Miyamoto et al., 2012). Our results that the same receptors were expressed both internally and peripherally suggests that individual receptors may receive nutritional signals from different locations, and then be subsequently integrated in the central nervous system to regulate food collection and feeding behavior. For example, sweet receptors in *D. melanogaster* are also found in different tissues; the detection of compounds by internal and peripheral sweet receptors are integrated in the central nervous system and control different aspects of feeding behavior (Yapici et al., 2016).

Our results suggest that antennae and mouthparts are major peripheral gustatory organs in bumble bees, in contrast to flies. We found the highest overall GR expression in the antennae and mouthparts. This pattern is different from what has previously been found in flies, where antennae are not a major gustatory organ, as they do not house gustatory receptor neurons (Clyne et al., 2000; Scott et al., 2001). We also found

relatively high expression of the antennal IRs in the antennae, but also detected expression of most of this subset of IRs in all of the other tissues. In addition to our molecular evidence, comparative bee morphology and behavior also support the idea that antennae are major taste organs in bees. Bees and other social hymenopterans have much longer antennae than flies (Snodgrass, 1935) and they are geniculate in form; this allows them to more directly contact important compounds in their environment, such as those arising from food or from social group members. Bumble bees progressively provision offspring, and feeding is elicited by chemical hunger cues from offspring (Den Boer & Duchateau, 2006). Further, bumble bees regularly inspect food stores with their antennae and respond to food storage levels (Dornhaus & Chittka, 2005). Our finding of widespread GR expression in antennae begins to shed light on the functionality of taste in bumble bees.

We also detected evidence of caste-specific patterns of expression for some genes examined in our study. The putative sugar receptor GR1, as well as IR93a, an antennal ionotropic receptor, and the two divergent IRs IR218 and IR339, were all expressed at higher levels in worker mouthparts compared to queen mouthparts. The difference in expression of these four genes in the mouthparts between these castes may suggest their involvement in feedback between nutritional cues in floral resources and brood as it relates to feeding and foraging behavior. There are widespread differences in queen and worker physiology, for example, queens live approximately one year, undergo diapause and reproduce, whereas workers only live several months and do not undergo any drastic physiological changes like diapause or reproduction. Our finding that some taste-related

receptors are also different between these two castes suggests that bumble bee caste differences also extend to taste perception, although the specific details of how they differ remain to be understood.

We detected expression of GRs and IRs in tarsi, but not at high levels compared to mouthparts and antennae, though we only sequenced the front tarsi. Despite low expression, we did find that there were significant differences in tarsal expression of some IRs between queens and workers. Specifically, IR76b and IR328 were higher in queen tarsi compared to workers, and IR93a, IR68a, IR75u, IR218, IR328, IR330, and IR331 were expressed at higher levels in worker tarsi compared to queens. IR76b receptors in the tarsi have previously been shown to be involved in amino acid detection in flies (Ganguly et al., 2017). The fact that this was expressed at higher levels in queen compared to workers suggests that queens may be able to detect nutrients that workers cannot, potentially nutrients that are important for offspring at the early nesting stage where queens exclusively feed brood. Sequencing of the other two tarsal pairs may demonstrate a greater role, or not, of the tarsi in bumble bee taste.

We detected high expression of several GRs and IRs in two internal tissues: the brain and the fat body. Specifically, we found high expression of the putative fructose receptor GR3 in the brain and fat body, and expression was higher in the worker relative to queen fat body. The GR3 ortholog Dm43a has also been detected in *D. melanogaster* brains and may be involved in the detection of fructose in the hemolymph and ultimately regulate feeding behavior (Miyamoto et al., 2012). GR3 and the IRs detected in the fat

body may be involved in the regulation of nutrient metabolism related to reproduction, which would explain differences between queens and the non-reproductive workers.

This is the first study to examine tissue- and caste-related gene expression of taste related genes in bumble bees. The extent to which the taste sensory modality is involved in bumble bee behavior is currently unknown, however a considerable amount of behavioral evidence suggests that it is important. Our results suggest that it may be playing an important functional role, as there are recent expansions in the GR gene family within the *Bombus* genus, and we found tissue and caste related differences in both GR and IR expression. We suggest that antennae and mouthparts are important taste tissues in bumble bees, and they should be targeted for future taste studies. Moreover, the fat body appears to be an important internal organ where nutrients are being regulated differently between queens and workers. Future studies should examine patterns of selection on these genes in order to help elucidate their functions. Our study ultimately provides strong candidate taste-related receptors for future mechanistic studies on taste that can shed light on the relevance of taste in the biology of this important pollinator group.

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Chapter Two: Worker task organization in incipient bumble bee nests

Abstract

Bumble bees (Genus: *Bombus*, Family: Apidae) are a longstanding model system for understanding animal behavior, ecology, and evolution. However, how workers in this system are organized to perform fundamental tasks related to brood feeding and food collection remains unclear. Bumble bees undergo dramatically different life stages, across which the social environment, and therefore task organization, changes over time. Queens initiate nests solitarily, and when the first cohort of workers emerge, they help the queen carry out brood feeding and food collection tasks, and she transitions to primarily egg laying. Although task organization has been studied in mature colonies, no studies to date have explored how these tasks are organized in young, incipient nests. Here, we explored how tasks related to brood feeding, food collection, and egg laying are organized by workers in incipient colonies. We found that bumble bee workers frequently switch between brood feeding and food collection tasks at this stage. Additionally, these tasks were nested, in that the majority of workers fed brood, a subset also collected nectar, and a smaller subset also collected pollen. This pattern suggests that the distinction between pollen collecting and non-pollen collecting might be the most important axis of division of labor in bumble bee nests, at least at the early nesting stage.

Introduction

A principal difference separating the social and solitary insects is that in the former, fundamental tasks related to feeding and reproduction are performed cooperatively by social group members, rather than by a single individual. All reproductive female insects, whether solitary or social, have to perform the fundamental tasks of finding food and suitable places to deposit and/or rear their offspring, in order to survive and reproduce. However, in the social insects, these tasks often change in both form and complexity, and are coordinated between multiple individuals that collectively perform a broad suite of tasks (Michener, 1969; Wilson, 1971). Identifying how social organization evolved in the insects is a major goal in social insect research (Korb & Heinze, 2008; Oster & Wilson, 1978; Toth & Rehan, 2017; Toth & Robinson, 2007; Wheeler, 1928).

Specialization, defined as individuals repeatedly performing the same task or task repertoire, to the exclusion of other tasks, is a prevalent component of social insect societies (Wilson, 1971). Specialization is considered adaptive for social groups because it can improve collective efficiency (Oster & Wilson, 1978); however, groups often perform more optimally when they contain both specialized and flexible individuals (Oster & Wilson, 1978). Social insects can vary dramatically with respect to the degree that they specialize on tasks. For example, some systems are organized by morphological or age-related polyethism, where worker age or morphology are associated with specialization on specific tasks (Mildner & Roces, 2017; Wilson, 1971). Other species have more flexible social organization, such as the annually or facultatively eusocial

species (Brian, 1952; West-Eberhard, 1967), where individuals often move fluently along a gradient between behavioral specialization and flexibility (Wilson, 1971).

Social insect research has historically focused heavily on systems in which task organization is highly predictive, and workers fall into discrete behavioral categories, such as in the honey bee *A. mellifera* (Robinson, 1987; Toth, 2005) and some ant species (Mildner & Roces, 2017; Wilson, 1971). The tractability of categorizing individuals into discrete behavioral groups has facilitated substantial insights into how tasks are organized. For example, in honey bees, worker tasks are organized around food-related tasks, whereby workers undergo an age-related transition from specialization on in-hive tasks, including brood feeding, to foraging (i.e., food collection). In this system, the categorical terms "nurse", and "forager" are applicable because they are alternative behavioral states. The nurse-forager dichotomy in honey bees has been used as a model to understand the underlying mechanisms of worker behavior in this and other social insect systems (Smith et al., 2008). Specifically, individuals can be grouped into discrete behavioral categories, and then compared for differences in physiology or gene regulation. This can be a helpful starting point to understand how task organization evolved and operates because it enables discovery of underlying mechanisms of task performance. In more flexible systems, however, overlaying such a dichotomy on task organization might be misleading in lineages where discrete behavioral categories might not exist. Studies that track individual behavior and quantify relative task performance, exclusivity (i.e., how many individuals perform a given task, and how evenly it is divided

among those individuals), and repeatability, are necessary to more fully comprehend task organization in more flexibly social systems.

Systems with more flexible task organization include the annually social species (Michener, 1969), wherein tasks may be organized in different ways at various life history stages. For example, the annually eusocial bumble bees undergo dramatically different life stages, across which the social environment, and therefore task organization, changes over time. Bumble bees collect pollen and nectar resources to progressively provision brood, similar to honey bees. However, in bumble bees, food-related tasks are exclusively performed by a solitary foundress queen when a nest is first initiated. After the first cohort of workers eclose in the nest, they assume brood feeding and food collection tasks, whereas the queen transitions to specializing on egg laying and ultimately ceases foraging and brood-feeding (Woodard et al. 2013; Shpigler et al. 2013). Examining worker task organization at this early stage enables the study of incipient and dynamic task organization at a time in nest development when queens still perform some food-related tasks, and then cease performing them through time. This can provide insights into how task organization develops in the nest, and also how it evolved and is maintained in this lineage. Additionally, given the small group sizes at this time (typically a queen and ~5 workers; Woodard et al. 2013), small changes in worker number equate to relatively large differences in group size, which might influence patterns of task organization (Dornhaus et al., 2012). This stage is also more experimentally tractable relative to larger (mature) colonies, which can have several

hundred individuals (Cnaani et al. 2002), because it is possible to perform relatively detailed behavioral observations on every group member.

The objective of this study was to determine how tasks that are essential to young nest growth and survival (food collection, brood feeding, and egg laying) are organized in the early nesting stage in bumble bees. For this, we used small, artificially assembled groups of the common eastern bumble bee, *Bombus impatiens*, that contained a queen and either three or five workers, or three or five workers without a queen. First, we examined how brood feeding and food collection tasks are organized amongst individuals by measuring how exclusive each task is. We also examined whether the performance of either of these tasks is related to egg laying. Next, we explored whether individual bumble bee workers consistently perform multiple tasks, or whether they tend to specialize on one task. Task organization and specialization have previously been quantified in bumble bees using various different frameworks, which can influence how individuals are characterized. To account for this in our analyses, we explored assigning bees to task groups using two different frameworks. The first method categorized individuals based on a single task they performed (Amsalem et al., 2014; Shpigler et al., 2016; Woodard et al., 2014), whereas the second method incorporated multiple tasks an individual performed in order to better accommodate task switching (Jandt et al., 2014).

Methods

Rearing and experimental design

Fifteen mature *B. impatiens* colonies (consisting of a queen and > 50 female workers) were acquired from Koppert Biological Systems (Howell, MI, USA) and maintained in their commercial boxes at the University of California, Riverside at 23°C and 40% RH. Colonies were fed *ad libitum* mix-source, honey bee-collected pollen purchased from Brushy Mountain Bee Farm (Moravian Falls, NC) and a syrup solution provided by Koppert Biological Systems. Individual bees were removed from source colonies to create small, artificially-constructed groups (hereafter "nests") in plastic nest boxes (15 x 15 x 10 cm) with the following four social configurations: queenright with three workers (QW3; n = 14 nests), queenright with five workers (QW5; n = 15), queenless with three workers (W3; n = 16), and queenless with five workers (W5; n = 13). *B. impatiens* queens initiate nests by laying a single cohort of typically five female-destined eggs (Leza et al., 2018; Watrous et al., 2019), then colonies grow over the course of the season, before producing reproductive offspring. We examined the impact of queen presence on workers (by manipulating the presence or absence of a queen) because bumble bee queens have direct inhibitory effects on worker egg laying behavior (Alaux et al., 2004; 2006); we might expect them to influence additional worker behaviors, based on the breadth of queen effects that have been observed in other social insect systems (Gamboa et al., 1990; Keller & Nonacs, 1993; Reeve & Gamboa, 1987; Traynor et al., 2014); and because they can themselves participate in the tasks we observed, early in colony development (Woodard et al. 2013; Shpigler et al. 2013).

For our two queenright groups (QW3 and QW5), callow queens (< 24 hours old; identified by their silvery appearance and inability to fly) were removed from their source colonies, maintained in small, plastic rearing containers (7 x 7 x 5 cm), and kept in a queen-rearing room that was temperature- and humidity-controlled at 25°C and 60% RH. All queens were unmated so that they would produce only haploid (male-destined) brood. This was done to make queenright groups more comparable with the queenless groups so that they would only produce male offspring, and to minimizing variation introduced by mating (Baer & Schmid-Hempel, 2005). Queens were treated with CO_2 gas at adult ages 12 and 13 days (30 minutes per day) to cause them to bypass diapause and initiate egg laying (Roseler, 1985). Queens will undergo reproductive maturation irrespective of mating status, and unmated queens that are subjected to CO_2 treatment develop their ovaries, lay eggs on a similar timescale as mated queens (Amsalem et al., 2015; Woodard et al., 2019), and suppress worker reproduction (Amsalem & Grozinger, 2017) equally well as mated, post-diapause queens. Workers were not subjected to this CO_2 treatment because they do not diapause, and, in the queenless groups with the absence of social inhibition, lay eggs approximately seven days after eclosion (Cnaani et al., 2002).

After the second CO_2 treatment, queens were either placed with three (to create QW3 nests) or with five (for QW5 nests) unrelated, callow workers in plastic queen rearing boxes purchased from Biobest USA, Inc. (15 x 15 x 10 cm). Queenless groups were also created with either three (W3) or five (W5) callow workers in identical rearing boxes. All workers were < 24 hours old at the time of group formation to prevent rejection by the queen (if applicable) and to control for age-related differences in

behavior among workers. We excluded extremely large- and small-bodied workers from these groups in order to minimize extreme differences in body sizes between nests. Our intention here was to increase statistical power and eliminate the need for unrealistic sample sizes required for highly variable size configurations. The body sizes of the workers included in this study were normally distributed, which is consistent with full size colonies (Couvillon et al., 2010), and had marginal wing cell lengths ranging from 1.85-3.25 mm. The lengths of the marginal wing cells are highly correlated with body size (Owen, 1988; Shpigler et al., 2013). The approximately two-fold size difference in bees in our study (see below) is comparable to the range found in young, newly formed nests (Shpigler et al., 2013; Watrous et al., 2019). Workers and queens were individually number-tagged (Betterbee, Greenwich, NY) using superglue as an adhesive, in order to track individual behaviors. Workers within a single nest originated from the same source colony, with equal representation from ten source colonies across social configurations. Queens originated from six different source colonies with equal representation across social configurations. Queens and workers in each experimental nest originated from two different source colonies.

All nests were maintained in a dark, temperature- and humidity-controlled (25°C, 60% RH) room with infrared cameras (VIGICA Peashooter QD520) placed directly above each nest to continuously record in-nest behaviors. Each nest was provided with a synthetic nectar solution that does not spoil readily (recipe provided in Boyle et al., 2018) to minimize handling of the feeders during the experiment, and both a waxed and unwaxed pollen ball (same pollen as described above, given a*d libitum*).

Nests were monitored daily for egg laying. Five days after eggs were observed in a nest, which is when eggs hatch into larvae (Cnaani et al., 2002), food resources were removed from inside the nest box. At this point, two separate, lighted (12:12 L:D cycle) food collection arenas were connected to the nest boxes via 30 cm translucent, polypropylene tubes (1.6 cm diameter). Nest boxes were covered in opaque black cloth such that nests remained dark (with the exception of any light entering through the tube), whereas the food collection arenas alternated between light and dark in a windowed room with supplementary artificial light during the day. Food was placed in clear, 6 oz close containers (Diameter = 7 cm). One food collection arena contained pollen (as described above, ground to a powder) and the other contained synthetic nectar (as described above) available through a cotton wick. Pollen was replaced every 4-5 days and synthetic nectar was replenished as needed and replaced every two weeks to avoid spoilage. Additional cameras were placed above each food collection chamber and continuously recorded activity until the first adult males eclosed (~25 days later). Bees in our experiment did not free forage but instead collected food from discrete foraging chambers. This allowed us to detect individual differences in food collection, while minimizing differences in the foraging environment experienced by individuals in free foraging colonies. Our experimental design allowed us to identify workers that were positively phototactic and motivated to collect food, which are two fundamental components of foraging, without introducing variation due to flight.

Nests were minimally disturbed during the ~25-day experimental period, with the exception of replacing pollen, nectar, or deceased workers. Any workers who died during

the experiment were replaced with callow workers (from the same source colony as the deceased bee), and the date of replacement was recorded. Nests were typically inspected daily, or at minimum every three days, so any worker replacement occurred within 24-72 hr of mortality. Nests in which the queen died, or more than two workers died ($N = 11$), were removed from the experiment and excluded from analyses (final sample sizes are reported in results). For each nest, on the date of first male adult eclosion, the entire nest (including any queens, workers, males, and brood) was frozen over dry ice and subsequently stored in a -80°C freezer.

Data collection and behavioral methods

Videos were analysed using Behavioral Observation Research Interactive Software (BORIS; Friard & Gamba, 2016) to quantify egg laying, brood feeding, and food collection (pollen or nectar) behaviors. We chose these four fundamental behaviors because they are compulsory to a successful colony; each must be performed in a nest that produces offspring. Egg laying was identified within videos as point events with no duration, and bee identity and the time and date of each event was recorded (Table 1; see example in Supplementary Video 1). Both queens and workers lay eggs, although queens primarily lay eggs in the queenright groups, and only workers lay eggs in the queenless groups. Given that egg laying events are relatively infrequent, we scanned videos and documented a minimum of three egg laying events per nest, including one or more events in the first five days of egg laying and also one or more additional events occurring a minimum of 10 days after the first recorded egg laying event.

To record events of brood feeding and food collection (pollen or nectar), 24 non-consecutive hours were randomly selected over each of two timeframes: 7-9 and 13-15 days after eggs were observed in the nest (hereafter referred to as the "early" and "late" timeframes). These timeframes capture (1) the time wherein the oldest larvae are 2-4 days old and bees had just begun carrying out brood care tasks in their newly-formed social groups, as well as (2) a later-stage time frame approximately halfway through the development of the first set of brood (Cnaani et al., 2002). At the late timeframe, the oldest larvae and pupae are 8-10 days old and nests typically contain larvae at all stages of development, and may also contain early stage pupae (Leza et al., 2018; Watrous et al., 2019). At this second timeframe, workers had been carrying out brood care tasks for more than one week. Randomly, selected hour long-observation periods were selected from a larger 24-hour cycle to encompass behavioral differences related to circadian rhythmicity (Yerushalmi et al., 2006). Because brood feeding occurs more frequently than food collection trips, based on our preliminary observations, we watched and scored more food collection video than in-nest video, in an effort to control for large differences in numbers of observations among behaviors. For each randomly selected hour, we observed the first five minutes of in-nest video and the entire hour of video of the food collection arenas. We assume that bees located in the food collection arenas were collecting the types of floral rewards they contained, given that all nests continued to successfully develop and grow, which requires pollen and nectar (Heinrich, 2004). However, we excluded data for any bees who remained within the food collection arenas for <10 seconds, in an effort to filter out exploratory or other non-food collection

behaviors in these arenas. All brood feeding events were recorded (bee identity, time, and date) as point events with no duration, and all putative pollen collection and nectar collection events were recorded (bee identity, time, and date) as state events with a duration (10s), start time, and stop time. Descriptions of these behaviors are provided in Table 1 and in Supplementary Videos 1-2. Total durations of in-nest observations were chosen based on previous studies that examined the frequency of these events in bumble bees (Shpigler et al., 2016; Woodard et al., 2014).

BEHAVIOR	**DEFINITION**
Brood feeding	Brood feeding is a stereotypical, discrete behavior (lasting 1-5 seconds) where individuals open a wax-covered larval cell and regurgitate into it by contracting their abdomen (described further in Free and Butler, 1959; Woodard et al. 2013).
Egg laying	Egg laying is a stereotypical, discrete behavior where the abdomen tip in placed inside an open wax cup, with legs gripping and sliding over wax. Eggs can be observed within the egg cup following this behavior.
Nectar collection	Here defined as when a bee was completely inside of the nectar chamber for 10 seconds.
Pollen collection	Here defined as when a bee was completely inside of the pollen chamber for 10 seconds.

Table 2.1*: Recorded behaviors with associated descriptions.*

Following nest collections, we measured several additional factors that might impact behavior in these nests, to include in our statistical analyses. We dissected brood cells over dry ice to quantify the number of eggs, larvae, and pupae in each nest box. The amount of brood in a nest influences the frequency and organization of egg laying, brood feeding, and food collection tasks (Kraus et al., 2019; Nagari et al., 2019; Orlova et al., 2019; Starkey et al., 2019; Woodard et al., 2013). We dissected worker ovaries in cold

100% ethanol, and the largest terminal oocyte in each ovary was staged (I-IV) according to groupings in Duchateau and Velthuis (1989) (hereafter "ovary stage"). Ovary stage is based upon the relative sizes of oocytes and their associated trophocytes and is independent of body size. We then recorded the binary (yes/no) resorption status of the largest terminal oocyte on each ovary. Hymenopteran females will commonly resorb nutrients from mature oocytes they cannot or do not oviposit, and resorption can be reliably identified based on the yellow, misshapen appearance of oocytes (Duchateau & Velthuis, 1989). Bees may resorb oocytes for a variety of reasons, such as social inhibition of oviposition or limited resources (Duchateau & Velthuis, 1989; Medler, 1962). We also quantified body size for all bees by removing the forewings and mounting them onto microscope slides, then measuring the marginal cell lengths with an ocular micrometer. We measured cells from both forewings and averaged the values together (hereafter this is referred to as "body size").

Statistical methods

All statistical methods were carried out in R (v. 4.0.0). Plots were generated with the ggplot package (v. 3.3.0, (Wickham, 2016)). For all statistical models, the best fit model was selected based on the lowest Akaike's Information Criterion for small sample sizes (AICc), using the model.sel() function from the car package (v. 3.0-7 (Fox & Weisberg, 2019)). The model with the lowest AICc score that was not rank deficient was selected for analyses. To control for differences in the relative frequency of observed behaviors, as well as differences in duration of behaviors, carried out in each nest (e.g., brood feeding is inherently more common than egg laying; brood feeding is a point event

and food collection is a phase event), we scaled counts of all behaviors according to their relative frequency using the following equation:

$$1 \text{ scaled count of behavior } Y = \frac{1}{\frac{\text{total counts of behavior } Y \text{ in the nest}}{\text{total counts of all behaviors in the nest}}}$$

In this way, the total scaled counts of each behavior in a given nest (i.e., scaled number of brood feeding, nectar collection, pollen collection, and egg laying events in each nest) were equal, and frequent behaviors did not dominate infrequent behaviors in our statistical analyses. All subsequent analyses were performed on scaled counts unless otherwise specified. All data filtering was performed on raw counts of behaviors. Egg laying was excluded from all time-specific analyses because the collection of egg laying data did not directly correspond with the two time frames during which other behaviors were observed.

Exclusivity of tasks in nests

We first quantified the exclusivity of each task within each nest, which we defined as the number and frequency of individuals observed carrying out a given task in a given nest. For this, we calculated Shannon diversity indices for raw counts of all tasks in nests using the diversity() function from the vegan package (v. 2.5-6, (Oksanen & Blanchet, 2019)), which has previously been used as a task specialization index (Gorelick et al., 2004). We only included nests with a minimum of three observations each of food collection, egg laying, and brood feeding (N = 33 nests). We chose this minimum threshold based on previously reported thresholds in the literature (Charbonneau &

Dornhaus, 2015; Shpigler et al., 2016) and to avoid drawing conclusions about tasks in nests with limited data. Here, the Shannon index incorporates the diversity and relative frequency of individuals carrying out each task in a given nest, thereby calculating each task's degree of exclusivity. A Shannon value of 0 indicates a highly exclusive task that was observed being carried out by only a single individual in the nest, whereas a high Shannon value indicates a task that many or all individuals in a nest were observed performing with relatively equal frequency. We compared Shannon indices among tasks to identify the most and least exclusive behaviors in nests with a generalized linear mixed model (GLMM) using the glmer() function from the lme4 package (v. 1.1-23, (Bates et al., 2015)), including the number of recorded instances of the task, social configuration, task identity, and the interaction between social configuration and task identity as possible fixed effects. Natal colony (i.e., the mature colony workers were collected from) and nest identity were included as random effects in all possible models.

Individual task organization

Specialization of individuals: Queens were excluded from all individual-based behavioral analyses (here and below) as our goal was to specifically understand worker task organization in the early nesting stage. To quantify the degree of behavioral specialization among individual bees (i.e., whether individuals specialized in performing only one or a few tasks), we calculated Shannon diversity indices on scaled counts of observed behaviors for all worker bees with a minimum of three raw behavioral observations (N = 121 bees). Here, the Shannon index incorporates the diversity and relative frequency of all tasks we observed each individual carry out, thereby calculating

each individual's degree of behavioral specialization. We then compared Shannon indices among social configurations to explore whether group size or presence of the queen influenced the degree of specialization of individual bees. We used a two-part GLMM including the number of scaled behaviors carried out by the individual and social configuration as possible fixed effects. Natal colony and nest identity were included as random effects in all possible models. We used a binomial distribution in part one of the model to analyze specialization as a binary response variable comparing perfectly specialized individuals (with a Shannon index of 0) to non-perfectly specialized individuals (with a Shannon index > 0). We used a gaussian distribution in part two of the model to analyze all non-perfectly specialized individuals along a continuous scale.

Single-task frameworks

We first quantified task organization among individual workers based on a single, predominant behavior we observed them carry out, using two approaches previously implemented in social insect research. We also included a framework that categorized individuals to a task if they performed it at least once. Only worker bees with a minimum of three raw behavioral observations (N = 121 bees) were included in these categorization analyses.

Most frequently performed task: We categorized worker bees into behavioral groups based on the scaled behavior they were observed performing most throughout the observation period (Shpigler et al., 2016).

Perfect specialization: Worker bees who were observed performing a single task exclusively were categorized as perfect specialists on the behavior they carried out.

Tasks performed at least once: Worker bees who were observed performing any task at least once were labelled as performing that task. In this last analysis, individual bees could be assigned to more than one category.

Multi-task frameworks

To establish behavioral categories that better incorporate the variation and flexibility among individuals in this system, we also categorized bees with three additional methods that incorporated all recorded behaviors carried out by each individual, rather than a single behavior. Here, we included all worker bees ($N = 179$), including those with fewer than three observations, because this set of methods can incorporate data-deficient bees with few (<3) behavioral observations without assigning task specialization to these individuals.

K-means categorization: First, we used a k-means clustering analysis (Hartigan, 1975; Ramette, 2007) on the scaled counts of observed behaviors carried out by each individual worker, to determine whether individual bees naturally cluster into distinct behavioral categories. K-means assigns individuals to k clusters with the lowest possible within-cluster variance. An elbow plot was used to determine the number of distinct clusters (k) that maximizes explanatory power while minimizing overfitting, and the kmeans() function defined k distinct clusters and assigned all bees to one of these clusters.

Shannon-based categorization: Next, we categorized worker bees based on their degree of specialization as calculated by the Shannon index, which incorporates the diversity and relative frequency of all tasks we observed each individual carry out, thereby capturing more of the continuous and complete behavioral performance of each bee. Here, we

labelled bees with a Shannon index below 0.6 to be “specialists”. Bees who met this criterion were said to specialize on the scaled behavior that they carried out most frequently. We also included a “generalist” bee category to represent bees who never specialized on a single task. Bees with a Shannon index above 0.6 were labelled “generalists”. We chose a threshold of 0.6 because the most frequently observed behavior comprised 71-100% of all behaviors carried out by specialists and 33-76% of all behaviors carried out by generalists. Bees with fewer than 3 raw observed behaviors were labelled as “other”.

For each method in both the single-task and multi-task frameworks, we used poisson-distributed generalized linear mixed models (GLMMs) to test whether the number of individual workers in a nest assigned to each behavioral category was influenced by social configuration. We included the number of individuals assigned to each category per nest as a response variable and behavioral category, social configuration, and the interaction between behavioral category and social configuration as possible fixed effects. Natal colony and nest identity were included as random effects in all possible models.

NMDS clustering: To visualize how individual workers cluster around behaviors, we performed a non-metric multidimensional scaling (NMDS) analysis on the scaled counts of observed behaviors carried out by each individual using the metaMDS() function from the vegan package using Euclidean distance. Here, we only included worker bees with three or more raw recorded behavioral observations ($N = 121$). NMDS takes the rows of a

multidimensional matrix (here, individual bees), and plots them in two-dimensional space to enable the visualization of multidimensional data.

Correlations between behavior and worker characteristics: To test whether any correlations existed between worker task specialization and body size or ovary development, in any of our behavioral frameworks, and whether these associations were consistent between frameworks, we used gaussian-distributed GLMMs. We compared body size and ovary development across behavioral categories for each framework. These models included average oocyte stage, resorption of ovaries, or body size as a response variable and behavioral category as a possible fixed effect. Natal colony and nest identity were included as random effects in all possible models.

Repeatability across time: We tested whether the behaviors we observed were performed consistently and repeatably across time, both within individual workers and within nests. We tested the repeatability of behaviors using scaled counts of behaviors, NMDS coordinates, and individual Shannon indices across time.

Task repeatability: To estimate the repeatability (R) for the scaled counts of each behavior, we used repeatability mixed models (RMMs) in the rptR package (Stoffel et al., 2017), a typical approach to measure behavioral repeatability. We did this at the individual, nest, and nest configuration levels to compare between the early to late timeframes. Here, we only included workers that had at least three raw observations at both timeframes ($N = 44$). All models were constructed with a gaussian error distribution for scaled counts of each behavior, with timeframe as a fixed effect and with natal colony, social configuration, and bee identity as random effects; we interpreted a

behavior as repeatable if the 95% confidence intervals of the random effect did not reach 0 (Stoffel et al., 2017). R is defined as the total variation that is reproducible among repeated measurements of the same individual (Nakagawa & Schielzeth, 2010).

Average Shannon repeatability: To investigate whether the average degree of specialization was consistent over time, we recalculated Shannon indices for individual worker bees based solely on the scaled behaviors carried out at a given timeframe (early or late) for each bee with at least three behavioral observations at one or both of these timeframes (n=108). We then compared the average Shannon index at each timeframe using a two-part GLMM including time frame (early or late) as a possible fixed effect. Individual bee within nest identity within social configuration, natal colony, and number of scaled behaviors were included as random effects in all possible models.

Change in Shannon repeatability: Additionally, to quantify change in specialization over time for individual worker bees, we calculated the difference between the early and late timeframe Shannon indices for each bee with at least three raw observations at both the early and late timeframes ($N = 44$ bees). We then performed a GLMM on this difference in Shannon index to identify any associations between social configuration and change in degree of specialization over time. Here, we used a gaussian distribution and included social configuration as a possible fixed effect. Natal colony and nest identity were included as random effects in all possible models.

ANOSIM: Finally, to determine whether timeframe influenced NMDS clustering, we performed an analysis of similarity (ANOSIM) on NMDS coordinates between the two timeframes.

Results

Our data analyses included a total of 43 nests (QW5 = 12, QW3 = 10, W5 = 9, W3 = 12), after removing 11 nests in which the queen died or >2 workers died from the experiment. We viewed and scored a total of 484.1 hours of video data (43.6 hours of in-nest video and 440.4 hours of food collection video) equally distributed across the 43 colonies, for an average of 1 hour (+/- 0.1 s.e.m.) of in-nest video and 10 hours (+/- 0.9) of food collection video per nest. Across all workers and queens in the experiment, we recorded a total of 1096 nectar collection, 315 pollen collection, 487 brood feeding, and 157 egg laying events (with a mean +/- s.e.m. per nest = 28.1 +/- 3.7 nectar collection, 14.3 +/- 3.4 pollen collection, 12.2 +/- 1.6 brood feeding, 4.6 +/- 0.4 egg laying). Social configuration predicted the amount of brood in the nest at the end of the experiment and the frequency of nectar and pollen collection (Table S1); thus, the factor termed "social configuration" in our statistical analyses encompasses these differences, in addition to differences in group size and/or the presence of the queen. Within the 43 analyzed nests, 12 worker bees from 10 nests died and were replaced. Replacement bees had fewer recorded behaviors than original bees (mean +/- s.e.m. = 5.1 +/- 1.7 behaviors per replacement bee (bees that were used to replace workers that died) versus 9.7 +/- 1.0 behaviors per original bee), and none of the 12 replacement bees were observed laying eggs. Scaling all individual behaviors to the total behaviors in each nest resulted in 1741 nectar collection, 1285 pollen collection, 1976 brood feeding, and 1617 egg laying scaled events (mean +/- s.e.m per nest = 40.5 +/- 0.8 nectar collection, 29.9 +/- 0.9 pollen

collection, 45.9 +/- 0.9 brood feeding and 37.6+/- 0.9 egg laying). All subsequent results reflect analyses on scaled behavioral counts unless noted otherwise.

Exclusivity of tasks in nests

Shannon indices of raw counts of behaviors in each nest (here measuring the degree of exclusivity of the tasks themselves) ranged from 0 (where only a single individual was observed carrying out that task) to 1.73 (where all individuals in the colony were observed performing that task multiple times) (Fig 2.1). Frequency of task performance (i.e., the number of times we observed a given task being performed by queens and workers) was positively correlated with Shannon index (GLMM: estimate = 0.005 +/- 0.001, $t = 4.61$, $N = 530$, $p < 0.001$). Egg laying and pollen collection were performed more exclusively than brood feeding and nectar collection (GLMM pairwise Tukey contrasts: brood feeding - egg laying estimate = 0.62 +/- 0.040, $z = 15.33$, $p < 0.0001$; nectar collection - egg laying estimate = 0.33 +/- 0.050, $z = 6.78$, $p < 0.0001$; pollen collection - brood feeding estimate = -0.58 +/- 0.059, $z = -9.89$, $p < 0.0001$; pollen collection - nectar collection estimate = -0.30 +/- 0.06, $z = -4.77$, $p < 0.0001$). Brood feeding was the least exclusive behavior, with 64% of all bees and 82% of non-data-deficient bees observed feeding brood at least once. In some instances, there was an interaction between social configuration and task identity. W5 nests had higher brood feeding Shannon indices than QW3 nests (GLMM Tukey contrast: estimate = 0.37 +/- 0.123, $t = 3.02$, df = 47.3, $p = 0.021$). Generally, nectar collection was more specialized in nests with fewer individuals (GLMM pairwise Tukey contrasts: W5 - W3 estimate = 0.77 +/- 0.12, $t = -6.4$, df = 46.8, $p < 0.0001$; W3 - QW3 estimate = -0.39 +/- 0.12, $t =$ -

3.21, df = 62.6, p = 0.011; W3 - QW5 estimate = -0.71 +/- 0.12, t = -6.15, df = 59.1, p < 0.0001; W5 - QW3 estimate = -0.38 +/- 0.12, t = -3.13, df = 47.3, p = 0.015; QW5 - QW3 estimate = 0.327 +/- 0.12, t = 2.79, df = 57.2, p = 0.035), though there was no difference between the two largest group sizes (W5 and QW5) (GLMM pairwise Tukey contrast: p = 0.96). The best fit model to predict task Shannon index included the frequency of task performance and the interaction between task identity and social configuration as fixed effects.

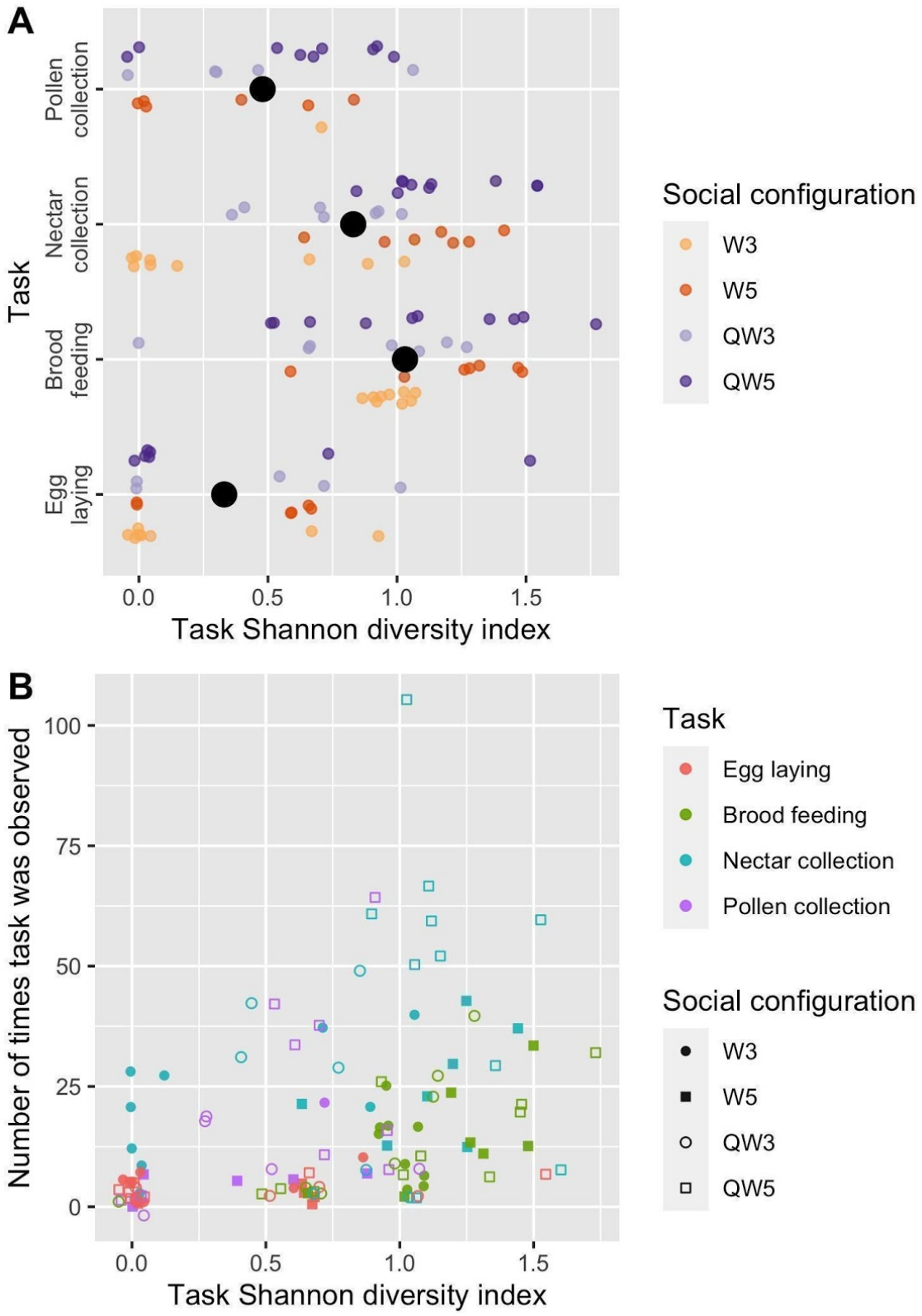
A
Task
Pollen collection
Nectar collection
Brood feeding
Egg laying
0.0
0.5
1.0
1.5
Task Shannon diversity index
Social configuration
W3
W5
QW3
QW5
B
Number of times task was observed
100
75
50
25
0
0.0
0.5
1.0
1.5
Task Shannon diversity index
Task
Egg laying
Brood feeding
Nectar collection
Pollen collection
Social configuration
W3
W5
QW3
QW5

Figure 2.1: Shannon diversity index for tasks in nests. A) Shannon task diversity for each task based on social configuration; W3 = Three Workers; W5 = Five Workers; QW3 = Queen + Three Workers; QW5 = Queen + Five Workers. Black dots represent mean Shannon diversity across all groups. Egg laying and pollen collection tasks were performed with more exclusivity (i.e., by fewer observed individuals) than nectar collection and brood feeding tasks (GLMM $p < 0.0001$). Brood feeding was more exclusive (lower Shannon values) in W5 nests than QW3 (GLMM $p = 0.02$), though they were still the least exclusive behavior in all groups. Nectar foraging Shannon decreased with increasing nest size (GLMM pairwise comparisons $p < 0.035$), with the exception that W5 and QW5 nests were not different from each other (GLMM $p > 0.1$). B) Frequency of observed task performance significantly predicted Shannon index (GLMM $p < 0.001$), whereby tasks observed being performed more frequently were less exclusive (had higher Shannon values) than those observed being performed less frequently. Points are jittered for easier visualization of overlapping points (width +/- 0.05; height +/- 2).

Individual task organization

Specialization of individuals: 120 of 179 worker bees met the threshold of at least three raw recorded behavioral observations and were included in the specialization analyses. Shannon indices of individual bees ranged from 0 (i.e., observed carrying out a single task exclusively; $N = 20$ out of 120) to 1.32 (e.g., observed carrying out all behaviors with similar frequency) (Fig 2.2A). Bee Shannon index did not differ based on social configuration (GLMMs $p > 0.1$; Fig 2.2B), but it could be partially explained by the number of observed behaviors for a given bee. Specifically, bees with fewer observed behaviors were more likely to be perfect specialists (have a Shannon value of 0) than those with more observed behaviors (Part 1 GLMM: estimate = -0.12 +/- 0.036, $z = -3.38$, $p = 0.0007$; not included in Part 2 GLMM best fit model; Fig 2.2C). The best fit model for part 1 included the number of behaviors and social configuration as fixed effects; the best fit model for part 2 was the null model.

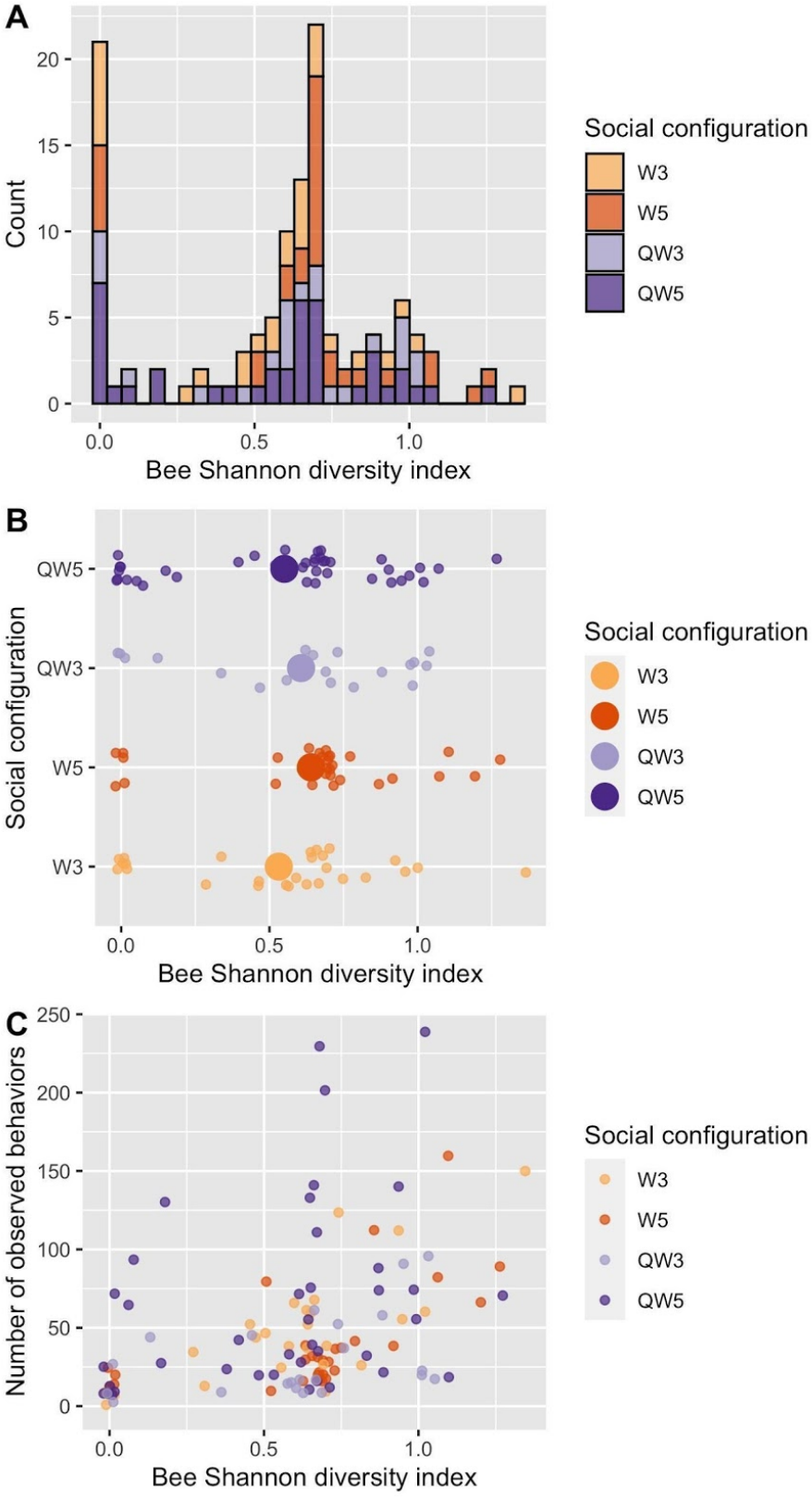
A
Count
20
15
10
5
0
0.0
0.5
1.0
Bee Shannon diversity index
Social configuration
W3
W5
QW3
QW5
B
Social configuration
QW5
QW3
W5
W3
0.0
0.5
1.0
Bee Shannon diversity index
Social configuration
W3
W5
QW3
QW5
C
Number of observed behaviors
250
200
150
100
50
0
0.0
0.5
1.0
Bee Shannon diversity index
Social configuration
W3
W5
QW3
QW5

Figure 2.2: Shannon diversity index of individual bees. A) Histogram of Shannon indices for all worker bees, coloured by social configuration; W3 = Three Workers; W5 = Five Workers; QW3 = Queen + Three Workers; QW5 = Queen + Five Workers. Bees exhibited a wide range of degree of specialization, and Shannon index did not differ by social configuration (this factor was not included in best fit model). B) Distribution of Shannon indices for all bees. Larger points indicate group means. C) Relationship between Shannon index and number of observed behaviors. Bees with fewer observed behaviors were more likely to be perfect specialists (have a Shannon value of 0) than those with more observed behaviors (Part 1 GLMM: estimate = -0.12 +/- 0.036, z = -3.38, p = 0.0007; not included in Part 2 GLMM best fit model). Individual points (not means) are jittered for easier visualization of overlapping points (width +/- 0.02: B height +/- 0.2; C height +/- 3).

Question	Method	Data	# bees in each category							Correlates	
			# bees included in analysis of 179 total	Egg layers	Nectar collectors	Pollen collectors	Brood feeders	Generalists	Other	Body Size	Ovary Developm-ent
Are frameworks based on a single behavior informative to describe task organization?	2.1) task performed most frequently	scaled counts of behaviors for workers with >= 3 raw observations	120 (40)	18 (17)	38 (31)	16 (15)	48 (30)	NA	NA	No patterns	No patterns
	2.2) perfect specialization	scaled counts of behaviors for workers with >= 3 raw observations	20 (15)	1 (1)	8 (6)	0	11 (9)	NA	NA	No patterns	No patterns
	2.3) task performed at least once	scaled counts of behaviors for workers with >= 3 raw observations	120 (40)	34 (24)	89 (36)	37 (21)	98 (38)	NA	NA	No patterns	No patterns
Are frameworks based on all recorded behaviors informative to describe task organization?	3.1) k-means clustering	scaled counts of behaviors for all workers	179 (43	0	0	12 (10)	0	167 (44)	0	Pollen collectors larger than	No patterns
	3.2) shannon-threshold- based specialization	shannon values based on scaled counts of behaviors for all workers	179	7 (7)	17 (15)	0	25 (17)	71 (34)	59 (28)	No patterns	No patterns

Table 2.2: Summary of task organizational framework analyses. Values represent number of worker bees assigned to each cluster; number in parentheses represent number of nests in which those bees were observed. K-means clusters did not directly correspond to these categories. For the purposes of this table, clusters were subjectively assigned to behavioral categories based on behavioral repertoire of bees in each category.

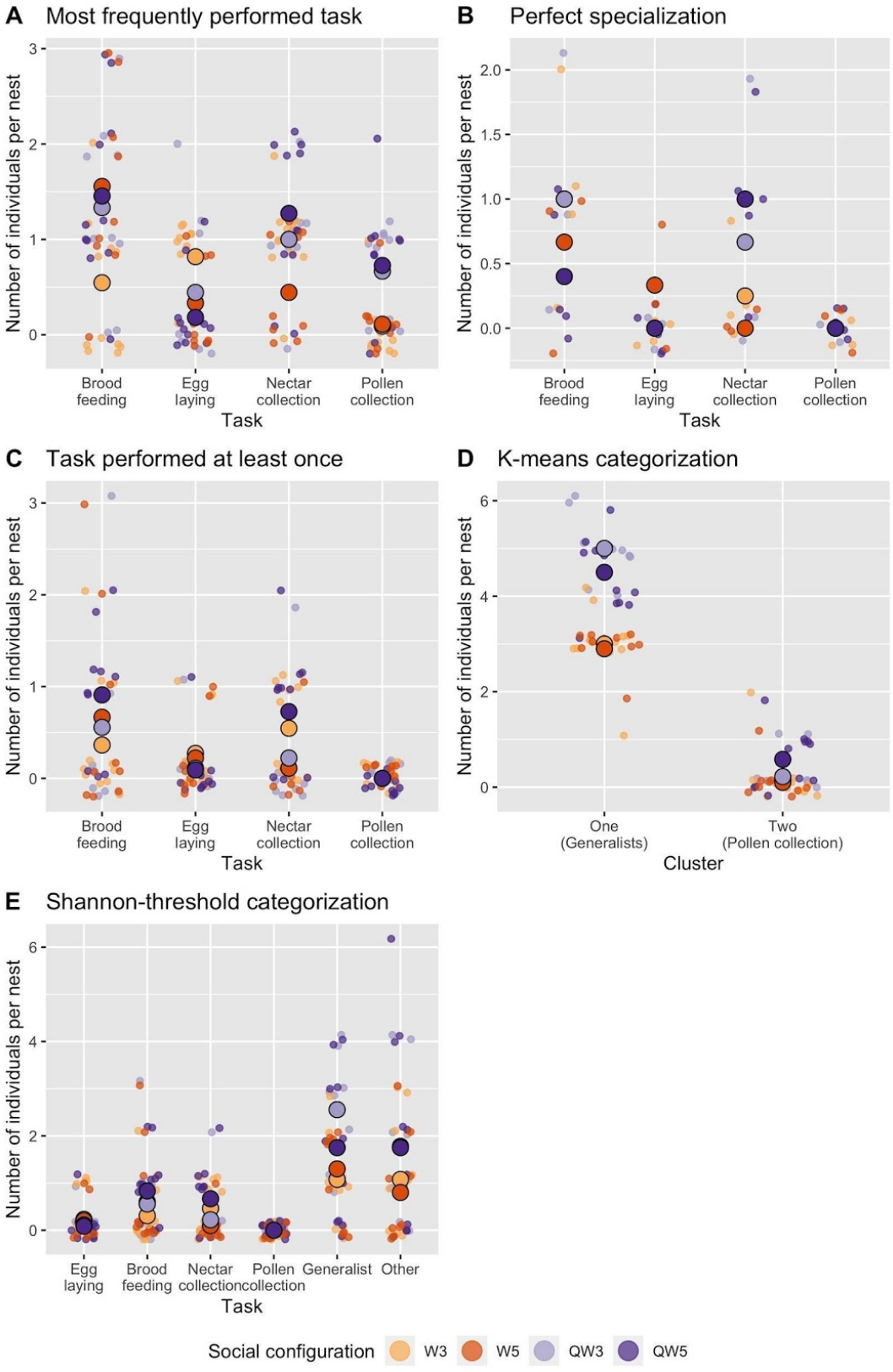
A Most frequently performed task
B Perfect specialization
C Task performed at least once
D K-means categorization
E Shannon-threshold categorization
Number of individuals per nest
Task
Brood feeding
Egg laying
Nectar collection
Pollen collection
Cluster
One (Generalists)
Two (Pollen collection)
Generalist
Other
Social configuration
W3
W5
QW3
QW5

Figure 2.3: Number of individual workers in a nest assigned to each behavioral category. Smaller points indicate a single nest and larger points indicate means for each social configuration; W3 = Three Workers; W5 = Five Workers; QW3 = Queen + Three Workers; QW5 = Queen + Five Workers. Individual points (not means) are jittered for easier visualization of overlapping points (width +/- 0.02: B height +/- 0.2; C height +/- 3).

Single-task frameworks

Most frequently performed task: Of the 120 worker bees from 40 nests that were observed performing at least three behaviors, 38 (from 31 unique nests) were classified as nectar collectors, 16 (from 15 nests) as pollen collectors, 48 (from 30 nests) as brood feeders, and 18 (from 17 nests) as egg layers, based on the scaled task they were observed performing the most (Table 2.2). Across all nests, significantly fewer individuals per nest were classified as pollen collectors compared to nectar collectors and brood feeders, regardless of nest social configuration (GLMM pairwise Tukey contrasts: nectar collection - pollen collection estimate = 0.87 +/- 0.30, $z = 2.90$, $p = 0.019$; brood feeding - pollen collection estimate = 1.10 +/- 0.29, $z = 3.81$, $p < 0.001$; Fig 2.3A). The best fit model, here predicting the number of individuals per nest carrying out a given task most frequently, included behavioral category alone as a fixed effect.

Perfect specialization: Of the 120 worker bees from 40 nests that were observed performing at least three behaviors, 20 (from 15 nests) were perfectly specialized, meaning that all of their observed behaviors were the performance of a single task. Eight (from 6 unique nests) were classified as nectar collectors, 0 as pollen collectors, 11 as brood feeders (from 9 nests), and 1 as egg layers (from 1 nest; Table 2.2). Neither social configuration nor behavioral category predicted the number of individuals categorized as specialists according to this method (GLMM behavioral category $p > 0.1$; social configuration not included in best fit model; Fig 2.3A). The best fit model predicting the number of individuals per nest perfectly specialized on each task included behavioral category alone as a fixed effect.

Tasks performed at least once: Of the 120 bees from 40 nests that were observed performing at least three behaviors, 89 bees (from 36 unique nests) were observed collecting nectar, 37 (from 21 nests) collected pollen, 98 (from 38 nests) fed brood, and 34 (from 24 nests) laid eggs (Fig 2.3C). Neither social configuration nor behavioral category predicted the number of individuals categorized as specialists according to this method (GLMM behavioral category $p > 0.1$; social configuration not included in best fit model; Fig 2.3E). The best fit model predicting the number of individuals per nest carrying out each task at least once included behavioral category alone as a fixed effect.

Multi-task frameworks

K-means categorization: Two behavioral clusters emerged that separated workers along a pollen collection axis (Fig 2.4). Workers who were observed collecting pollen >30 scaled times were assigned to cluster two (n = 12 bees from 10 nests), and those who were observed collecting pollen <30 scaled times were assigned to cluster one (n = 167 from 44 nests; Table 2.2). Brood feeding and egg laying did not appear to impact clustering (Fig. 2.4). Significantly more workers were categorized into cluster one (generalists) than cluster two (frequent resource collectors) (GLMM estimate = 2.63 +/- 0.30, $z = 8.81$, $p < 0.0001$). Social configuration did not predict the number of bees assigned to each cluster (GLMM $p > 0.05$). The best fit model predicting the number of individuals per nest assigned to each k-means cluster included behavioral category and social configuration as fixed effects.

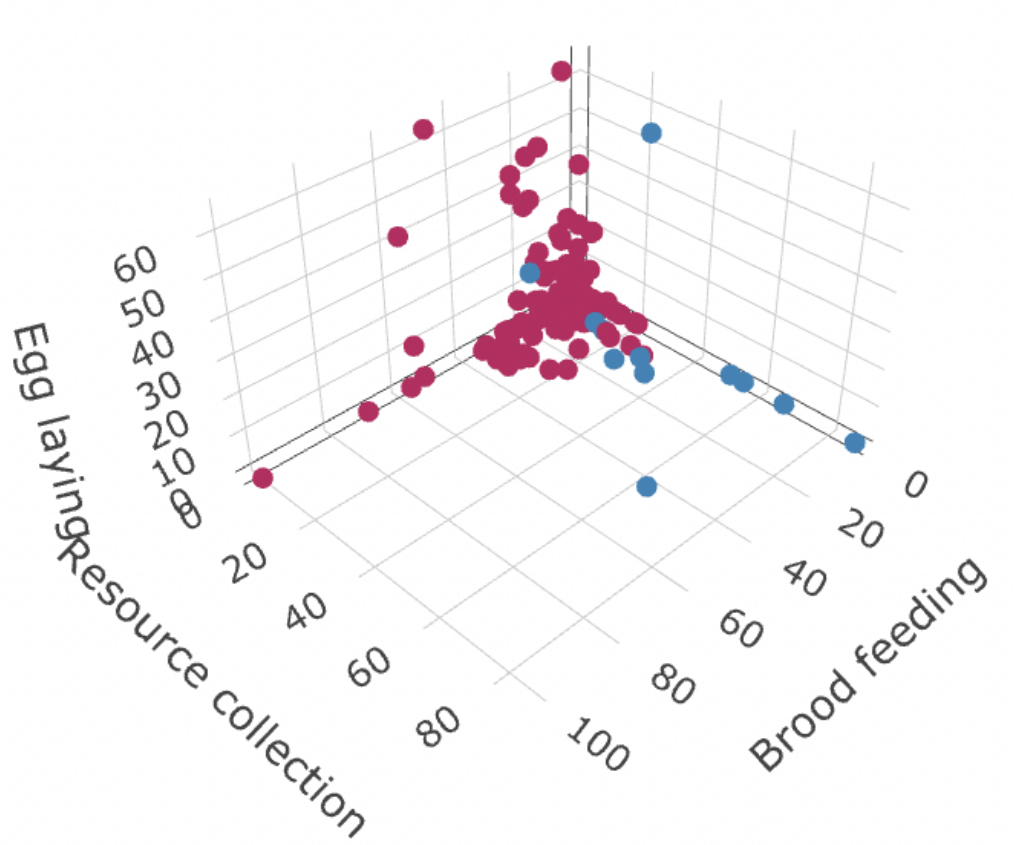

Figure 2.4: K-means clustering analysis. Colors represent clusters: maroon = one (generalists), blue = two (frequent pollen collectors). Resource collection axis is nectar and pollen collection events summed together for easier visualization, but these types of food collection behaviors were assessed separately in the analysis.

Shannon-based categorization: Based on a Shannon threshold of 0.6, we categorized 49 worker bees as specialists (Shannon ≤ 0.6; from 32 nests), 71 as generalists (Shannon > 0.6; from 34 nests), and the remaining 59 bees as other (<3 total observed behaviors; from 28 nests) (Table 2.2). Specialists were either egg layers (n = 7 from 7 nests), brood feeders (n = 25 from 17 nests), or nectar collectors (n = 17 from 15 nests). No pollen collection specialists emerged from this analysis. There were more generalists and other bees in each nest relative to bees specialized on brood feeding, egg laying, and nectar collection (all relevant GLMM Tukey contrasts $p < 0.001$). Social configuration did not significantly predict the number of individuals per behavioral category (GLMM $p > 0.1$). The best fit model predicting the number of individuals per nest assigned to each behavioral category based on a Shannon threshold of 0.6 included behavioral category and social configuration as fixed effects.

NMDS clustering: The NMDS analysis plotted individual bees across two major axes, with nectar and pollen collection clustering close together, brood feeding clustering near food collection, and egg laying as distinct from the other behaviors (Fig 2.5). NMDS1 described an egg laying - food collection axis, with brood feeding falling between these two other tasks. In NMDS2, egg laying and pollen collection were very similar, while brood feeding was more differentiated from the remaining three behaviors. Individuals fell at all points across the plot and did not display obvious, distinct clustering across these two axes.

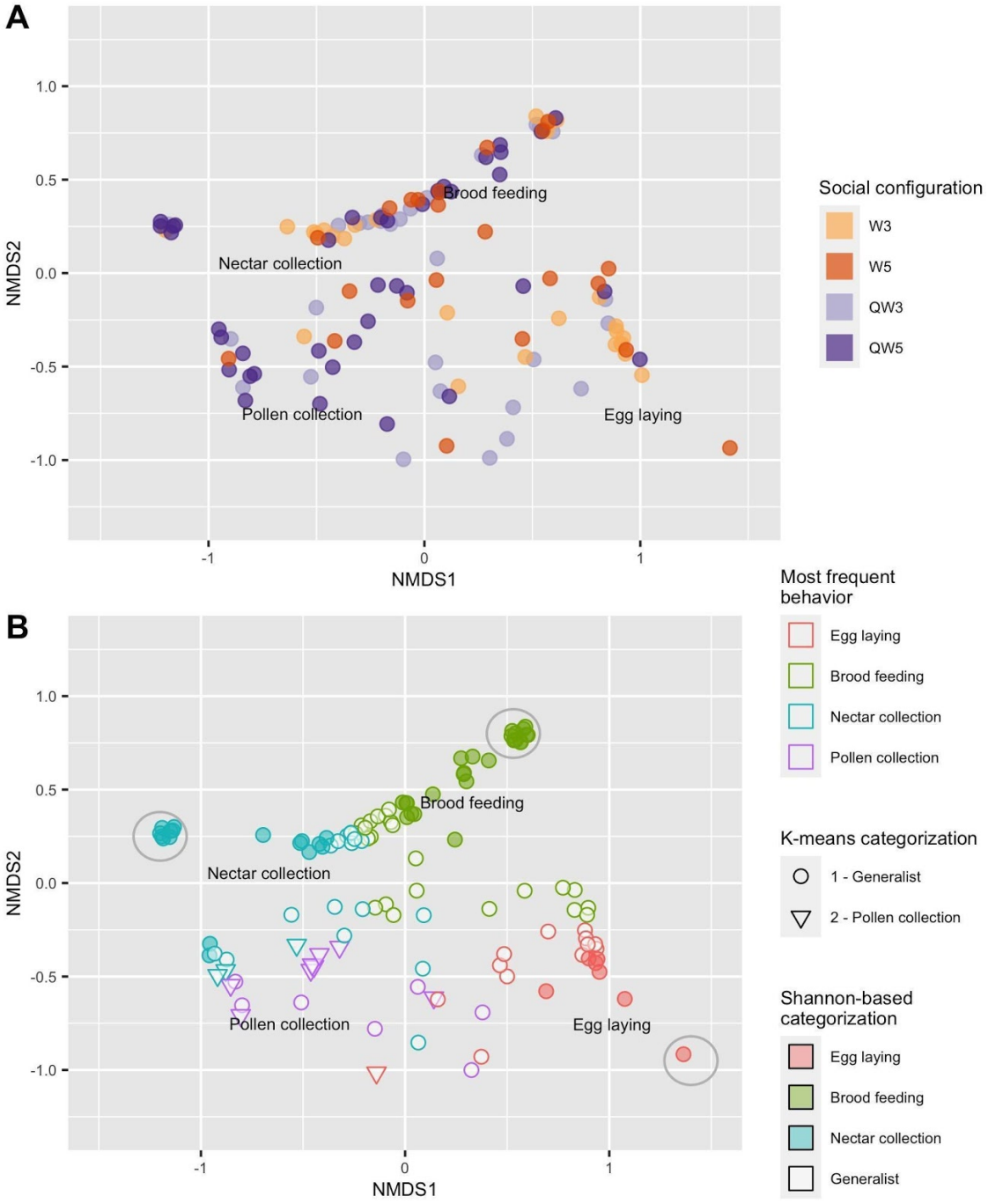
A
1.0
0.5
0.0
-0.5
-1.0
NMDS2
-1
0
1
NMDS1
Brood feeding
Nectar collection
Pollen collection
Egg laying
Social configuration
W3
W5
QW3
QW5
B
Most frequent behavior
Egg laying
Brood feeding
Nectar collection
Pollen collection
K-means categorization
1 - Generalist
2 - Pollen collection
Shannon-based categorization
Egg laying
Brood feeding
Nectar collection
Generalist

Figure 2.5: NMDS plots. Points represent individual worker bees. Task names are centered over their respective loci. Points are jittered to more easily visualize overlapping points (width +/- 0.05, height +/- 0.05). A) NMDS coordinates of individual bees coded by social configuration; W3 = Three Workers; W5 = Five Workers; QW3 = Queen + Three Workers; QW5 = Queen + Five Workers. B) NMDS coordinates of individuals coded by categorization method. Color and shape refer to the category of each bee from each of the four behavioral categorization methods. Large grey circles surround clusters of perfect specialists. There were no perfect pollen collection specialists. Bees with fewer than 3 raw behavioral observations are not included in this plot. Among these analyses, there was a high degree of variation: Not a single individual bee was sorted into the same category across all four categorization methods employed here.

Correlations between behavior and worker characteristics

In the k-means analysis, bees from cluster two (frequent pollen foragers) were, on average, larger-bodied than workers in cluster one (infrequent pollen foragers) (GLMM Tukey contrast: estimate = 0.23 +/- 0.068, $z = 3.43$, $p < 0.0001$). Cluster one contained a normal distribution of worker body sizes across the full range of body sizes (wing marginal cell length ~1.8 - 3.2mm), whereas cluster two only contained bees with marginal cell lengths > 2.6 mm. The best fit model to predict the body size of bees in the k-means analysis included behavioral cluster alone as a fixed effect. Behavioral category was not included in the best fit models for any other relevant analyses, indicating that none of the four behaviors observed could predict body size, ovary stage, or ovary resorption status in any of the remaining clustering or categorization analyses (Table 2.2; Fig 2.6). The null model was the best fit for all of these analyses.

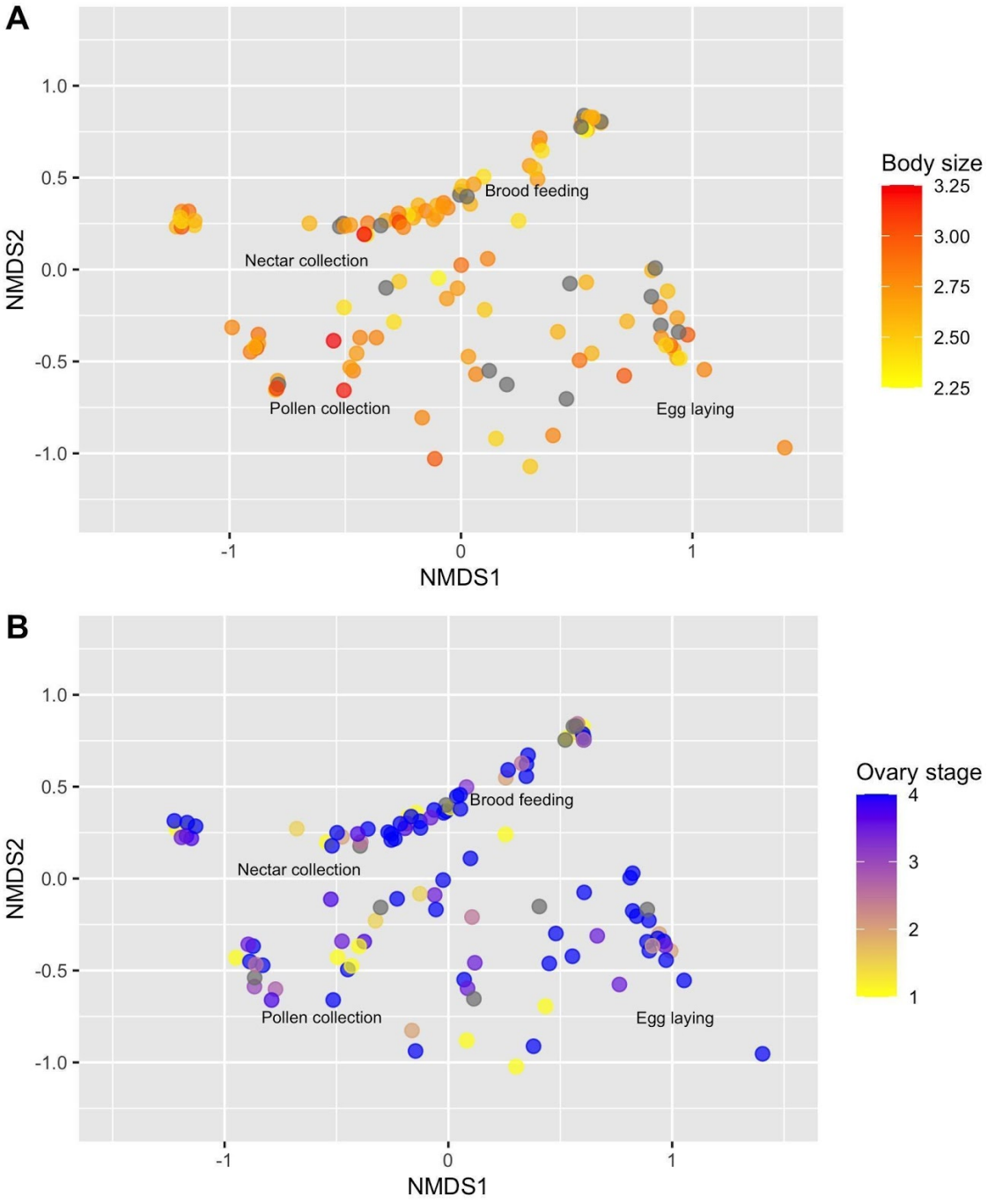

Figure 2.6: NMDS with worker body size and ovary stage. Grey points are missing data on body size or ovary stage. Task names are centered over their respective loci. Points are jittered to more easily visualize overlapping points (width +/- 0.05, height +/- 0.05). A) NMDS coordinates of individual worker bees based on body size. B) NMDS coordinates of individual worker bees based on ovary stage.

Repeatability across time

Task repeatability: Pollen collection was repeatable for individuals, but not nests or social configurations (Fig. 2.7). No other behaviors were repeatable for individuals, nests, or social configurations. This indicates that individuals change in both the frequency and repertoire of observed behaviors over time, with the exception of those collecting pollen. Nectar collection (RMM: R = 0 +/- 0.04, p = 0.5) and brood feeding (RMM: R=0 +/- 0.04, p= 1, Fig. 2.7B) occurred more frequently in nests, on average, during the later time frames.

Average Shannon repeatability: The degree of specialization of individuals did not change over time, as there was no change in mean Shannon index in bees from the early to the late timeframe (time frame was not included in best fit models). The best fit models to predict time-dependent Shannon index had no fixed effects and included only source colony and bee identity within nest identity within social configuration as random effects.

Change in Shannon repeatability: Individual change in Shannon index could not be explained by social configuration (social configuration not included in best fit model). The best fit model to predict an individual's change in Shannon index had no fixed effects and included only source colony and nest identity within social configuration as random effects.

ANOSIM: NMDS patterns also did not change with time frame based on our analysis of similarity (R=0.010; p=0.16), indicating that similar task repertoires were filled at the early and late timeframes (although not necessarily by the same individuals).


A
Pollen collection
Nectar collection
Brood feeding
Task
-50
0
50
Change in number of behaviors performed by each individual
B
Pollen collection
Nectar collection
Brood feeding
Task
-50
0
50
100
Change in number of behaviors performed by each nest
Social configuration
W3
W5
QW3
QW5
Percent change
0
500
1000
1500
>0 observations at both timeframes
yes
no

Figure 2.7: Repeatability Analysis. Individual points are jittered to more easily visualize overlapping points (width +\- 2, height +\- 0.3). A) Each point represents an individual worker bee, and the change in scaled number of observed behaviors from the early to late timeframe for each individual. No behaviors were repeatable across time. B) Each point represents a nest, and the change in number of observed behaviors from the early to late timeframe within each nest.

Animal Welfare Note

All bees were maintained under standard rearing conditions during the experiment with constant access to food resources. Bees were euthanized at the end of the experiment using dry ice, which is among the most humane methods of euthanasia. We worked only with commercially reared *Bombus impatiens* and thus did not negatively impact any wild populations. We made every effort to meet the high standards of animal welfare required by Animal Behavior for the Use of Animals in Research. We followed all legal requirements for working with *Bombus impatiens* and followed all institutional guidelines. Colonies were transported and maintained at the Insectary and Quarantine Facility at UC Riverside under California Department of Food and Agriculture permit number 3182.

We assembled small worker groups with unmated queens. This was amenable to increasing our sample size without requiring an excessive number of full-sized colonies. We based our sample size on preliminary experiments and previously published studies on bumble bee behavior.

Discussion

Identifying how task performance is organized in insect societies is a major goal in sociobiological research (West-Eberhard, 1967; Michener, 1969; Wilson, 1971; Oster & Wilson, 1978). We explored task organization in the early nesting phase of bumble bee colonies. Our goal was to investigate patterns of specialization and flexibility, and to explicitly document how food-related tasks, including brood feeding and food collection, are organized amongst individuals at this stage. In our examination of brood feeding and

food collection behaviors, as well as egg laying, we found that pollen collection and egg laying were more exclusive behaviors, in that they were often carried out by fewer individuals in the nest. In contrast, the majority of workers fed brood. With the exception of pollen collection, individual task performance was not repeatable across time, indicating that the task repertoire of individuals changes over time, at least for the approximately week-long period examined here. Workers tended to switch frequently between multiple tasks during the observation periods (two three-day timeframes). Further, bees exhibited a broad distribution of task specialization, as some individuals specialized on a single task, but most were more flexible in that they performed multiple tasks. Despite the value that can be gained from categorizing individuals within social insect nests, we found that in the early nesting phase in the bumble bee *B. impatiens*, task organization does not fit into a predictable, categorical framework, based on the tasks that we observed. This finding is consistent with previous bumble bee studies that examined mature colonies, which also detected considerable amounts of task switching amongst workers (Brian, 1952; Cartar, 1992; Jandt et al., 2009a).

The origin of eusociality is hypothesized to have been driven in part by the benefits of having multiple individuals care for offspring (Gadagkar, 1990; Korbe & Heinze, 2008) in which the reproductive individual's daughters help collect food and provisioning offspring. In many incipiently social and subsocial bees and wasps, there is little task division between helpers (Dew & Michener, 1981; Rehan & Richards, 2010; Wcislo & Gonzalez, 2006). In more socially complex species, however, brood feeding and foraging are generally uncoupled, such that a subset of individuals perform brood

feeding and another subset collect food resources from outside the nest (Free, 1955; Bassindale, 1955). These tasks can be associated with physiological factors such as body size, sucrose responsiveness, and ovary development (Amdam et al., 2004). In our study, we observed a nested pattern, in that most workers fed brood, a subset also collected nectar, and then a smaller subset also collected pollen. While this result related to foraging specialization is consistent with studies of mature bumble bee nests, which found evidence that subsets of workers specialize on pollen and nectar foraging (Goulson, 2002; Spaethe et al., 2007), our study demonstrates that these specialized subgroups may also feed brood when they are inside the nest. In-nest behaviors were not observed in the majority of these previous studies, with the exception of one, which also found that bees that forage also feed brood (Brian, 1952). Furthermore, the food-related behaviors we examined were not correlated to body size or ovary development in the majority of our analyses. These results suggest that bumble bee worker task organization at this stage may be more similar to more incipiently social insects, rather than lineages with more complex eusociality, such as honey bees or ants.

Our finding that most workers fed brood suggests that performance of this task is shared by most group members at this early stage in nest development, in contrast to many other advanced social insect systems (Free, 1955; Bassindale, 1955). Of the four behaviors we observed, brood feeding was the least exclusive behavior across all social configurations. 78% of non-data-deficient bees fed brood at least once, and brood feeders were the most common behavioral category based on the tasks that bees were observed performing most frequently. Based on this, it appears that all workers have a low

threshold for responding to the signals that elicit brood feeding behavior. Similar to honey bee larvae, bumble bee larvae require food continuously (Pereboom et al., 2003; Plowright & Pendrel, 1977), thus brood feeding behavior must occur frequently and consistently in the nest. However, brood feeding does not appear to be physiologically constrained in bumble bees as it is in honey bees, where nurse honey bees exclusively produce royal jelly, a key food source fed to honey bee larvae in addition to pollen (Snodgrass, 1925). Because there is no evidence for royal jelly production, or any other physiological constraint related to brood feeding, in bumble bees (Sadd et al., 2015; Drapeau et al., 2006; Kupke et al., 2012; Pereboom, 2000), workers that are in close spatial proximity to brood may instead be more likely to perform this behavior, rather than workers with a unique physiological propensity to do so (Jandt et al., 2000b; Nagari et al. 2019; Crall et al., 2018).

Although most bees in our study collected nectar (70% of bees), pollen collection emerged as a relatively exclusive behavior, as fewer (31% of bees) individuals performed this behavior. In our study, of the 108 bees who were observed collecting food resources, 3% collected pollen only, 62% collected nectar only, and 35% collected both. Similarly, individuals that collected pollen did so consistently across the observation period, which provides additional evidence that this a more specialized task. This pattern is generally consistent with previous studies in bumble bees (Cartar, 1992), which have demonstrated that a subset of bumble bee workers that forage exhibit long-term specialization on either pollen or nectar collection (Hagbery & Nieh, 2012; O'Donnell et al., 2000; Russell et al., 2017). Frequent pollen collectors were distinct from infrequent pollen collectors in the k-

means frameworks in our study, which is consistent with the idea that a subset of workers perform the majority of foraging trips, which has been shown in previous studies on bumble bees (Goulson et al., 2002; Russell et al., 2017), and has also been observed in honey bees (Gernat et al., 2018). Foraging is a cognitively demanding task for bees (Menzel, 2012), and pollen collection specifically has also been proposed to be a more complex and cognitively demanding task than nectar foraging (Heinrich, 2004; Muth et al., 2016). Further, unlike brood feeding, there is previous evidence from bumble bees that foraging is associated with unique behavioral and physiological characteristics, such as positive phototaxicity (Porath et al., 2019) and an increased density of olfactory sensilla (Spaethe et al., 2007). Based on these previous studies, and our own findings about the relative exclusivity of pollen collection, we propose that the distinction between pollen collecting and non-pollen collecting might be the most important axis of division of labor in bumble bee nests, at least at the early nesting stage.

Although pollen collection emerged as a relatively specialized behavior, all individuals who collected pollen were also observed performing other behaviors. Thus, frequent food collection did not preclude the performance of additional, in-nest behaviors. Further, we did not find strong evidence that pollen collection specialization was associated with body size. Previous studies on mature bumble bee colonies have identified that larger bees have a greater density of sensilla (Spaethe et al., 2007; Russell et al., 2017), which may contribute to a greater capacity to discriminate between olfactory cues in floral resources (Chittka & Raine, 2006). Similarly, some studies on mature colonies have found that larger bees are more specialized on pollen collection (Cartar,

1992; Goulson, 2002), although other studies have failed to find these associations (Smith et al., 2016; Russell et al., 2017). In our study, frequent pollen collectors were larger, on average, than infrequent pollen collectors, but this was based solely on the k-means framework. The remaining four out of our five analyses found no relationship between body size and pollen collection. Many large bees did not collect resources at all and there was substantial overlap in body size across groups. Although we purposefully limited body size variation within groups, which may have precluded finding task associations with body size, our data suggest that body size does not relate to propensity to collect pollen in young nests.

Egg laying was the most exclusive behavior we measured across all nests: only 28% of workers were observed laying eggs, primarily in the queenless groups. This finding is consistent with other studies of bumble bee worker reproduction in small groups, which have found that in the absence of the queen, a single worker typically emerges as a dominant egg-layer (Amsalem et al., 2013; Cnaani et al., 2007). In the queenright groups, all queens laid the majority of eggs, although some workers also laid eggs. More broadly, previous work has suggested that individuals are less likely to switch between performing a task that has a strong underlying physiological basis, such as those requiring changes in reproductive status (Johnson, 2005). However, nearly all of the egg laying workers in our study also performed other tasks. Individuals that laid eggs more commonly collected pollen, compared to collecting nectar.

In incipient bumble bee nests, there is a transitional period when the first cohort of workers emerge (~5 workers), and they begin to help the queen with food-related tasks

(Woodard et al. 2013; Shpigler et al., 2013). During this period, the queen typically continues to collect food and feed brood for some period of time, before she transitions to primarily egg laying. The ability of workers at the early stages of nest development to successfully collect food for the colony and feed offspring is pivotal for the nest to advance to a mature stage where reproductive individuals are produced (Malfi et al., 2019). However, interestingly, we detected almost no influence of group size or the presence of the queen on worker behaviors at the incipient nesting stage, with the exception that there was a reduction of worker egg laying in queenright nests. Because there are so few individuals in the nest at this stage, the addition or removal of a few individuals equates to large differences in total group size (e.g., a 100% increase in group size between our W3 and QW5 groups), which might be predicted to have observable effects on social organization. Queens in social insect colonies have also been proposed to act as "pacemakers" that regulate the behavior of workers (Kocher & Grozinger, 2011). Our data suggest that queens are not pacemakers in incipient bumble bee nests, at least in regards to food-related behaviors, in contrast to queens in other social insect colonies, as well as mature bumble bee colonies (Orlova et al., 2020). Rather, our findings are more consistent with what has been observed in *Polistes* wasps (Jha et al., 2006), a social insect system with an annually social lifestyle that is relatively similar to bumble bees. Our findings suggest that although queens play unique roles in young nests, their contribution to food-related tasks does not have a unique influence on worker behavior.

Additionally, we found that one third of bees in our study performed fewer than three recorded behaviors. This pattern, where a significant proportion of workers are observed carrying out few or no tasks, has also been observed in ants (Charbonneau & Dornhaus, 2015) and honey bees (Shpigler et al., 2017). These observations of inactivity for a subset of workers might be related to the importance of behaviorally plastic "replacement workers" (Hasegawa et al., 2016) for the long-term persistence of social insect colonies, which may even be relevant in small, incipient nests. However, in reference to all of our results, we caution that more direct comparisons of food-related behaviors in both incipient and mature colonies are needed to determine if these patterns are consistently observed as colonies develop. In species where colonies grow in size with season or age, like bumble bees, evidence of group size influences on task organization has been mixed and may only emerge when there are external ecological pressures like parasitism or competition for resources (Dornhaus et al., 2012), which were not present in our study.

Previous work with mature *B. impatiens* colonies has shown little evidence of in-nest worker specialization; instead, individuals frequently switch between tasks at random (Jandt et al., 2009a). The majority of bees in our study (56%) were observed performing more than one unique task, including during two relatively short (three-days each) timeframes. Our results thus build on these results from more mature colonies and suggest that regular task switching is also common in the early stages of the colony. Bees with more observed behaviors were more likely to be categorized as generalists, suggesting that the appearance of specialization in some bees may have been due solely

to a lack of observations. In this way, we likely overestimated the amount of specialization in these groups. The lack of consistent patterns among behavioral groups, and the lack of individual behavioral repeatability in our study, further indicate that task performance is not consistent or predictable in bumble bees. Based on the categorization framework we implemented, individuals were grouped into different behavioral categories. This subsequently impacts how the social organization in the bumble bee system is understood as well as the strength and accuracy of correlations between behavioral categories and underlying morphological and physiological features. Moreover, with the exception of one framework where we found pollen collection associated with body size, we did not find any associations with body size or ovary development with task, including egg laying. Workers can lay eggs and then resorb their ovaries, which explains this disparity (Duchateau & Velthuis, 1989). The lack of consensus among frameworks for worker behavioral categorization in our study suggests that, although we can place individuals into discrete categories based on, for example, their predominant task, doing so results in an underestimation of behavioral variation, which can result in misleading and uninformative conclusions. This may explain why previous attempts to categorize bumble bees based on performance of food-related behaviors have failed to uncover any underlying physiological and molecular factors associated with these tasks (Cameron, 1989a; Cameron & Robinson, 1990; Couvillon et al., 2011; Smith et al., 2016). Based on this, we propose that individual bumble bee workers cannot be reduced to discrete task groups based on their performance of food-related tasks.

Our study demonstrates the importance of using a more holistic framework that captures multiple behaviors a single individual performs, as well as how the organization of these behaviors changes at different colony stages (Costa et al., 2021). We found that workers in the incipient stage perform multiple food-related tasks, and variation between workers appears to be based on whether they engage in complex tasks like food collection, in addition to basic tasks like brood feeding, rather than these tasks being uncoupled between subsets of workers. Behavioral frameworks like individual response threshold models (Beshers & Fewell, 2001) or animal personality (Jandt et al., 2013; Walton & Toth, 2016), that incorporate multiple behaviors that an individual performs, may better explain the variation in task performance we observed in our study. These more holistic analyses of behavior are consistent with a growing shift towards incorporating social complexity in behavioral research, rather than reducing it (Holland & Bloch, 2020). Such a continuous framework enables researchers to describe where individuals fall along a behavioral spectrum, without using discrete labels, and can enable the discovery of co-regulated behaviors (Jandt et al., 2013). This information can then be leveraged to correlate behaviors with potential molecular, physiological, and/or morphological characteristics that underlie individual behavior, if they exist. A more holistic framework that incorporates a multiple tasks performed by individuals can also be applied to other biological systems that demonstrate behavioral flexibility, as well as systems that do not, which can then be leveraged to compare task organization across social insect taxa.

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Chapter Three: Pollen nutrients: a key feature in plant-pollinator interactions

Abstract

Understanding the underlying nutritional drivers of floral visitation is especially compelling in generalist species, such as bumble bees, because they typically visit a broad suite of plant species. In this study, we used a metabolomic approach to explore associations between floral visitation, inferred from pollen collection behavior, and pollen nutritional composition in wild bumble bees. Specifically, we asked whether there are species-specific differences in pollen nutrient collection, and whether these patterns are also shaped by the floral resource environment. We found that diversity of metabolites was consistent across bumble bees in different environments, but that richness varied. We also found that the composition of nutrients varied based on species and ecoregion. This provides support that pollen nutrients may also drive bumble visitation to floral resources.

Introduction

In the field of pollination biology, the question of what drives floral visitation has always been a major organizing topic (Wasser & Ollerton 2006). Understanding what drives floral resource visitation is important because it can ultimately influence plant reproductive success, pollinator health, and the structuring of entire ecosystems (Woodard & Jha, 2017). Paradigms for understanding floral visitation have largely been developed and tested through the lens of higher-level ecological processes, such as pollinator sensory biology (Chittka & Raine, 2006), optimal foraging theory (Waddington & Holden 1979), and pollinator movement through complex landscapes (Jha & Kremen, 2012; Carvell et al., 2017). These frameworks have been critical for elucidating general patterns of floral visitation for pollinator species in different floral resource environments. However, it is still difficult to predict which flowers pollinators will ultimately visit. This is because many underlying mechanisms that drive floral visitation remain unresolved (Woodard & Jha, 2017).

Interactions between bee nutritional requirements, nutrient detection, and nutrient composition in floral resources have recently begun to be integrated into floral visitation paradigms (Vaudo et al., 2015; Woodard & Jha, 2017). This has largely been facilitated by molecular analyses of nutrition and has led to a greater mechanistic understanding of broader ecological patterns of floral visitation. Pollen nutrients include amino acids, lipids (sterols), secondary metabolites, and fatty acids (Roulston & Cane, 2000; Palmer-Young et al., 2019). Pollen is primarily collected to support offspring and adult reproduction. Nectar contains sugars and secondary metabolites that sustain the energy

demands of adults (Heinrich, 2004). The molecular composition of both of these floral rewards can vary considerably depending on plant species, and recent evidence suggests that bees can discriminate between nutrients in both pollen and nectar (Wright et al., 2013; Kessler et al., 2015; Ruedenauer et al, 2017; Lim et al., 2019). Thus, preference for specific nutrients may be a bottom-up driver of plant visitation (Somme et al., 2015; Stabler et al., 2015; Ruedenauer, 2016; Vaudo et al., 2016; Muth et al., 2016). Moreover, feedback between floral resources collected and colony development may drive repeated visitation to specific floral resources, or floral constancy, which is a cornerstone of effective pollination services (Chittka et al., 1999). However, at present, our nascent understanding of these processes is limited, and studies on the nutritional basis of wild bee behavior are exceedingly rare (Woodard & Jha, 2017).

In this study, we used a metabolomic approach to explore associations between floral visitation, inferred from pollen collection behavior, and pollen nutritional composition in wild bumble bees. Specifically, we asked whether there are species-specific differences in pollen nutrient collection, and whether these patterns are also shaped by the floral resource environment. Understanding the underlying nutritional drivers of floral visitation is especially compelling in generalist species, such as bumble bees, because they typically visit a broad suite of plant species. Thus, they can make decisions about which specific plant species they collect pollen and nectar from, in heterogeneous resource environments where choices are possible (Fowler et al., 2016). Further, bumble bee species can occupy considerably different floral resource environments throughout their range (Thorp et al., 1983). Within shared habitats,

different bumble bee species may either occupy unique (Cole et al., 2020) or shared (Goulson et al., 2010) floral niches. The social biology of bumble bees adds additional complexity to their foraging-related decision-making (Fischer & O'Connell, 2017). Bumble bees are eusocial and foraging adults navigate nutritional signals from their foraging environment (Chittka & Raine, 2006) to satisfy the complex nutritional needs of their colony (Kraus et al. 2019).

To test whether there is evidence that pollen nutrients drive floral resource visitation in bumble bees, we quantified relative amounts of nutrients in wild-caught bumble bee pollen loads using a metabolomics approach. Specifically, we collected pollen loads from foraging workers of two common species in the Sierra Nevada mountains, *Bombus vosnesenskii* and *B. melanopygus,* in four unique ecoregions throughout the region. Both species have overlapping ranges and visit similar plant species (Williams et al., 2014; Cole et al., 2020). Based on the hypothesis that nutrients in pollen influence pollen collection in heterogeneous floral resource environments where choices are possible, we predicted that there would be both species-specific and ecoregion-specific patterns in pollen nutrients. This is the first study to examine nutrients in wild-caught bumble bee collected pollen.

Methods

Site Selection and experimental design:

We collected 20 *B. melanopygus* workers and 25 *B. vosnesenskii* workers, and their pollen loads, across twelve meadows in four different ecoregions throughout the

Sierra Nevada Mountains of California to compare how differences in habitat type and species influences nutritional profiles in pollen. These two species are the most common in this region, with *B. vosnesenskii* being overwhelmingly the most relatively abundant bumble bee species in the Sierra Nevada (Loffland et al., 2017), and the entire state of California (Thorp et al., 1983; Fisher et al., *submitted*). Workers from both species were collected on the same day at each meadow, and thus had access to the same floral resources. This allowed us to examine species-specific patterns in pollen collection behavior. All workers were wild-caught and immediately stored in liquid nitrogen in the field. Pollen was removed from the corbicula (hind legs) in the lab and transferred to a -80°C freezer until metabolomic analysis. Workers were also kept at -80°C for a future study. Meadows were assigned to Environmental Protection Agency (EPA) Level IV ecoregions (Griffith et al., 2016) as a proxy for floral resource environment. Ecoregions are classified by in abiotic and biotic components of ecosystems, including vegetation; California has 177 Level IV ecoregions (Griffith et al., 2016), and we sampled in four of these. At each ecoregion, we collected at least three individuals from each species, with a mean of 5 ± 1.1 *B. melanopygus* workers per ecoregion and 6.3 ± 1.3 *B. vosnesenskii* workers per ecoregion.

Metabolomics analysis

Sample preparation: We used metabolomic analyses to explore the relative abundance of polar and nonpolar metabolites within each pollen load sample. Pollen samples were weighed and 200 μL of extraction solvent (20:20:30:30 IPA:water:ACN:MeOH) was

added per 10 mg. Samples were bead milled at 4 C, sonicated 30 min in an ice bath, then vortexed for 30 min at 4 C. After centrifugation for 15 min at 4 C at 16,000 x g, the supernatant was transferred to a glass autosampler vial and analyzed by LC-MS for targeted and untargeted analyses, performed at the UC Riverside Metabolomics Core Facility.

LC-MS metabolomics, untargeted: The untargeted analyses were performed on a Synapt G2-Si quadrupole time-of-flight mass spectrometer (Waters) coupled to an I-class UPLC system (Waters). Separations were carried out on a CSH phenyl-hexyl column (2.1 x 100 mm, 1.7 μM) (Waters). The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The flow rate was 250 μL/min and the column was held at 40° C. The injection volume was 1 μL. The gradient was as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 40% B; 24 min, 100% B; 26.5 min, 100% B; 27 min, 1% B. The MS was operated in negative ion mode (50 to 1600 m/z) with a 100 ms scan time. MS/MS was acquired in data dependent fashion, with 1 MS/MS scan per MS scan. Source and desolvation temperatures were 150° C and 600° C, respectively. Desolvation gas was set to 1100 L/hr and cone gas to 150 L/hr. All gases were nitrogen except the collision gas, which was argon. Capillary voltage was 2 kV. A quality control sample, generated by pooling equal aliquots of each sample, was analyzed every 4-5 injections to monitor system stability and performance. Samples were analyzed in random order. Leucine enkephalin was infused and used for mass correction.

LC-MS metabolomics, targeted: Targeted metabolomics of polar, primary metabolites was performed on a TQ-XS triple quadrupole mass spectrometer (Waters) coupled to an

I-class UPLC system (Waters). Separations were carried out on a ZIC-pHILIC column (2.1 x 150 mm, 5 μM) (EMD Millipore). The mobile phases were (A) water with 15 mM ammonium bicarbonate adjusted to pH 9.6 with ammonium hydroxide and (B) acetonitrile. The flow rate was 200 μL/min and the column was held at 50° C. The injection volume was 1 μL. The gradient was as follows: 0 min, 90% B; 1.5 min, 90% B; 16 min, 20% B; 18 min, 20% B; 20 min, 90% B; 28 min, 90% B. The MS was operated in selected reaction monitoring mode. Source and desolvation temperatures were 150° C and 500° C, respectively. Desolvation gas was set to 1000 L/hr and cone gas to 150 L/hr. Collision gas was set to 0.15 mL/min. All gases were nitrogen except the collision gas, which was argon. Capillary voltage was 1 kV in positive ion mode and 2 kV in negative ion mode. A quality control sample, generated by pooling equal aliquots of each sample, was analyzed every 3-4 injections to monitor system stability and performance. Samples were analyzed in random order.

Metabolite annotation: Data processing (peak picking, alignment, deconvolution, integration, normalization, and spectral matching) was performed in Progenesis Qi software (Nonlinear Dynamics). To aid in the identification of features that belong to the same metabolite, features were assigned a cluster ID using RAMClust[1]. Annotation level 1 indicates an MS and MS/MS match or MS and retention time match to an in-house database generated with authentic standards. Level 2a indicates an MS and MS/MS match to an external database. Level 2b indicates an MS and MS/MS match to the Lipiblast in-silico database[2] or an MS match and diagnostic evidence, such as the dominant presence of an m/z 85 fragment ion for acylcarnitines. Level 3 indicates an MS

match, though some additional evidence is required, such as adducts were detected to sufficiently deduce the neutral mass or the retention time is in the expected region. Several mass spectral metabolite databases were searched against including Metlin, Mass Bank of North America, and an in-house database.

Statistical analyses

All statistical analyses were performed with R (v. 4.0.0) and all plots were generated with the ggplot package (v. 3.3.0, (Wickham, 2016)). Only characterized metabolites were used for analyses so that functions could be deduced. Focal analyses on amino acids, sterols, and fatty acids were also performed because of their known relevance for bumble bee colony health (Arien et al., 2015; Moerman et al, 2017; Vanderplank et al., 2020). For analyses with multiple comparisons, we used false discovery rate (FDR) corrections to adjust P-values, given the number of tests employed. *Diversity and richness of metabolites in pollen samples:* We calculated (1) total richness, the number of metabolites, and (2) Shannon diversity, the abundance and evenness of metabolites, for each pollen load using the specnumber() and diversity() functions in the Vegan package (Oksanen et al., 2020). We tested whether variation in total metabolomic richness and diversity could be explained by ecoregion and/or species using a generalized linear mixed model (GLMM) using the glmer() function from the lme4 package (v. 1.1-23, (Bates et al., 2015)). We generated a global model with diversity and richness as response variables; ecoregion, species, and their interaction as fixed effects; and meadow as a random effect with a gaussian distribution. We then sequentially removed fixed

effects from the global model and selected the model that best fit the data based on the lowest Akaike's Information Criterion for small sample sizes (AICc), using the model.sel() function from the car package (v. 3.0-7 (Fox & Weisberg, 2019)). The model with the lowest AICc score that was not rank deficient was selected for analyses. We then performed Type II Wald chisquare tests on the best model post-hoc to test significance of factors.

Similarity in metabolites between samples: To visualize how similar pollen samples were based on nutritional content, we performed a non-metric multidimensional scaling (NMDS) analysis on the relative amounts of metabolites in each sample using the metaMDS() function from the vegan package using a Bray-Curtis distance dissimilarity index. This was followed by an analysis of similarity (ANOSIM) in the Vegan package to test whether there were differences in metabolite composition between ecoregions and species. The ANOSIM compares the mean distance between samples within an ecoregion, or species, to the mean distance among samples between ecoregions, or species, based on metabolite clustering patterns (Clarke, 1993). We performed the NMDS and ANOSIM on 1) the broad suite of all characterized metabolites, or targeted analysis of 2) amino acids, 3) fatty acids, and 4) sterols.

Focal metabolomic analyses: We tested whether variation in all individual amino acids, sterols, and fatty acids could be explained by ecoregion and/or species using generalized linear mixed model (GLMM) as above.

Results

Diversity and richness of metabolites in pollen samples: We characterized 63 polar metabolites and 208 non-polar metabolites in our dataset. For pollen collected by *B. melanopygus*, we found an average richness of 218.2 ± 1.9 metabolites and average diversity of 2.4 ± 0.02 metabolites. For pollen collected by *B. vosensenskii*, we found an average richness of 222.5 ± 1.7 metabolites and average diversity of 2.4 ± 0.02 metabolites (Fig. 3.1). Metabolite richness was higher in *B. vosensenskii* in general (GLMM: Estimate=10.32; CI: 3.59-17.06; $p < 0.01$), but higher in *B. melanopygus* collected pollen in the S. Sierra Lower Montane ecoregion (GLMM: Estimate=14.48; CI: 6.18-22.77 -22.77; $p < 0.01$). Metabolite richness did not differ within species in different ecoregions. Metabolite diversity did not differ between species or ecoregion, as neither of these factors were predicted to be in the best model.

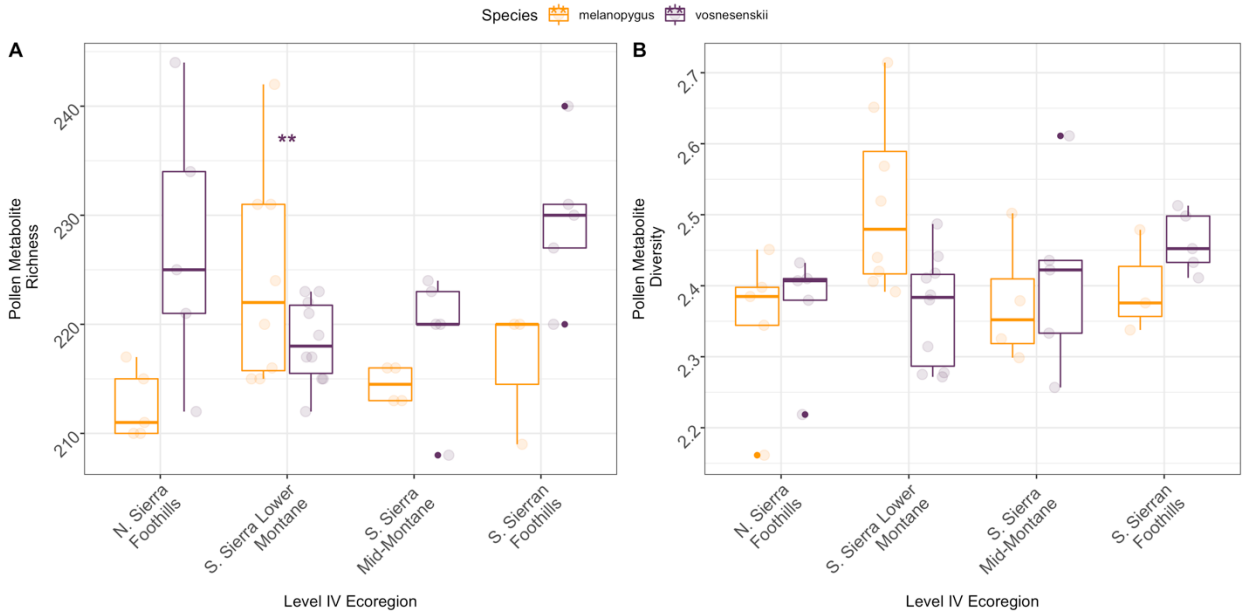

Figure 3.1: A) Metabolite richness of *B. vosnesenskii* and *B. melanopygus* collected pollen. Overall richness was higher in *B. vosnesenskii* collected pollen (GLMM: $p < 0.01$). Pollen metabolite richness was higher in *B. melanopygus* collected pollen in the S. Sierra Lower Montane (GLMM: $p < 0.01$). B) There was no difference in diversity of metabolites in pollen between ecoregions or species.

Similarity in metabolites between samples: There were differences in metabolite composition between species (ANOSIM Species: $R = 0.22$; $p < 0.001$) and ecoregion (ANOSIM Ecoregion: $R = 0.21$; $p < 0.001$) when all metabolites were considered (Fig. 3.2A; $R^2 = 0.98$; stress = 0.15). There was a difference in amino acid composition (Fig. 3.2B; $R^2 = 0.98$; stress = 0.13) between species (ANOSIM Species: $R = 0.11$; $p > 0.05$) and between ecoregions (ANOSIM Ecoregion: $R = 0.20$; $p < 0.01$). Sterol composition (Fig. 3.2C; $R^2 = 0.98$; stress = 0.15) differed in similarity between species (ANOSIM Species: $R = 0.14$; $p < 0.01$) and ecoregion (ANOSIM Ecoregion: $R = 0.24$; $p < 0.001$). Fatty acid composition (Fig. 3.2D; $R^2 = 0.99$; stress = 0.09) differed between species (ANOSIM Species: $R = 0.08$; $p < 0.05$) and ecoregion (ANOSIM Ecoregion: $R = 0.10$; $p > 0.05$).

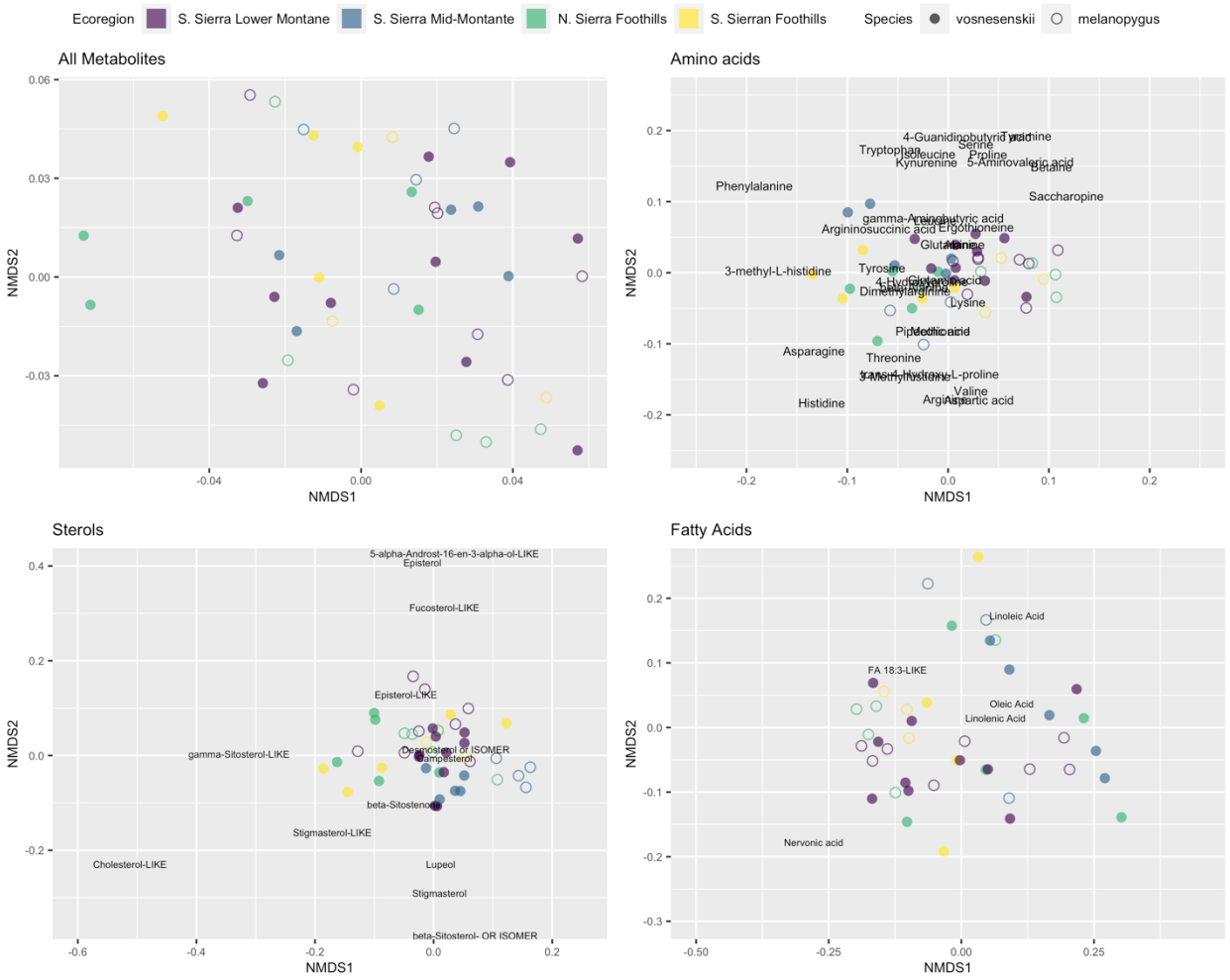

Figure 3.2: Non-metric multidimensional scaling of metabolites in pollen for each sample based on A) All metabolites; B) Amino acids; C) Sterols; and D) Fatty acids. Ordinations were based on Bray-Curtis dissimilarity. Points clustered closely together indicate shared similarity in metabolite composition based on classes of nutrients.

Focal amino acid analyses: There was a significant interaction between ecoregion and species that explained variation in relative amounts for amino acids for fifteen metabolites in pollen (Fig. 3.3A). Four amino acids differed between ecoregion (Fig. 3.3B), four amino acids differed between species (Fig. 3.3C), and fifteen did not vary between species (Fig. 3.3D). For example, arginine was found at relatively high levels in *B. melanopygus* collected pollen loads in the S. Sierra Mid-Montane ecoregions compared to *B. vosnesenskii* but was higher in *B. vosensenskii* collected pollen in the Southern Sierra Foothills compared to *B. melanopygus* (GLMM $X^2 = 44.3$; Df = 3, FDR corrected $p < 0.001$). Similarly, 4-hydroxyproline was found at higher levels in *B. melanopygus* collected pollen in the Southern Sierra Mid-Montane ecoregion and Southern Sierra Lower Montane but was higher in *B. vosnesneskii* in the Northern and Southern Sierra Foothills (GLMM $X^2 = 61.6$; Df = 3, FDR corrected $p < 0.001$). Both species collected more methionine in the S. Sierra Mid-Montane ecoregion (GLMM $X^2 = 9.8$; Df = 3, FDR corrected $p < 0.05$) and less serine in the Southern Sierra Lower Montane (GLMM $X^2 = 11.7$; Df = 3, FDR corrected $p < 0.05$) compared to other ecoregions. Leucine (GLMM $X^2 = 12.3$; Df = 1, FDR corrected $p < 0.01$) and tryptophan (GLMM $X^2 = 12.3$; Df = 1, FDR corrected $p < 0.001$) were found at higher levels in *B. vosnesenskii* collected pollen regardless of ecoregion. Finally, glutamine levels were high in all pollen loads but did not vary between species or ecoregions (FDR corrected $p > 0.05$).

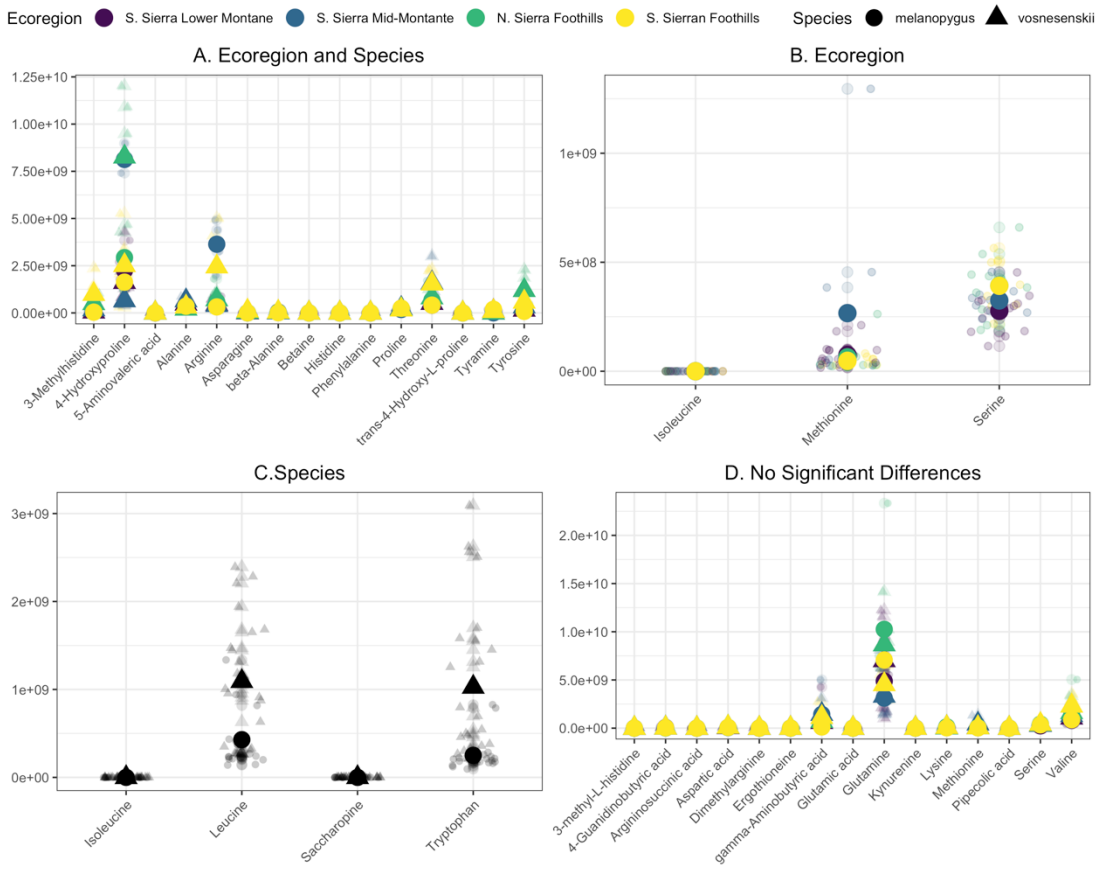

Figure 3.3: Relative abundance of amino acids in pollen. A) Amino acids that were significantly different between both ecoregion and species; B) Amino acids that were significantly different between ecoregion only; C) Amino acids that were significantly different between species only; and D) Amino acids that were not different between species or ecoregion. Individual points are jittered to more easily visualize overlapping points (width +\- 2, height +\- 0.3)

Focal sterol analyses: Beta-sitosterol (or its isomer) was higher in pollen collected in S. Sierra Mid-Montane compared to other ecoregions (GLMM $X^2 = 58.7$; Df = 3; FDR corrected $p < 0.001$) and *B. vosnesenskii* collected more beta-sitostenone than *B. melanopygus* (GLMM $X^2 = 11.6$; Df = 1; FDR corrected $p < 0.01$). There was a significant interaction between ecoregion and species that explained variation in relative amounts of five sterols (Fig. 3.4A) and six did not vary between ecoregion or species (Fig. 3.4B). For example, *B. vosnesneskii* collected more campesterol (GLMM $X^2 = 40.1$; Df = 3; FDR corrected $p < 0.001$) and stigmasterol (GLMM $X^2 = 60.1$; Df = 3; FDR corrected $p < 0.001$) in the Southern Sierra Mid-Montane compared to *B. melanopygus*. Fucosterol-like sterol and lupeol were present in all samples at high levels regardless of species or ecoregion (FDR corrected $p > 0.05$).

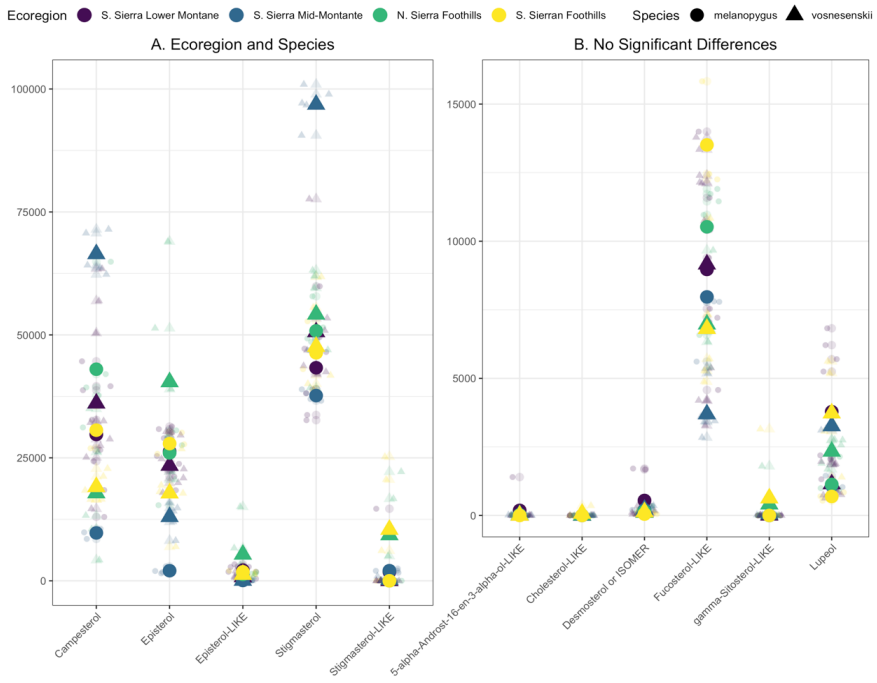

Figure 3.4: Relative abundance of sterols in pollen. A) Sterols that were significantly different between both ecoregion and species and B) Sterols that were not different between species or ecoregion. Individual points are jittered to more easily visualize overlapping points (width +\- 2, height +\- 0.3).

Focal fatty acid analyses: The relative amount of oleic acid was higher in pollen collected from the Northern Sierra Foothills compared to pollen from other ecoregions (GLMM $X^2 = 20.7$; Df = 3; $p < 0.01$), but amount was low compared to linolenic and linoleic acid, which were especially high in all pollen regardless of species or ecoregion (Fig. 3.5). Relative amounts of the remaining four fatty acids did not differ between species or ecoregion (FDR corrected $p > 0.05$).

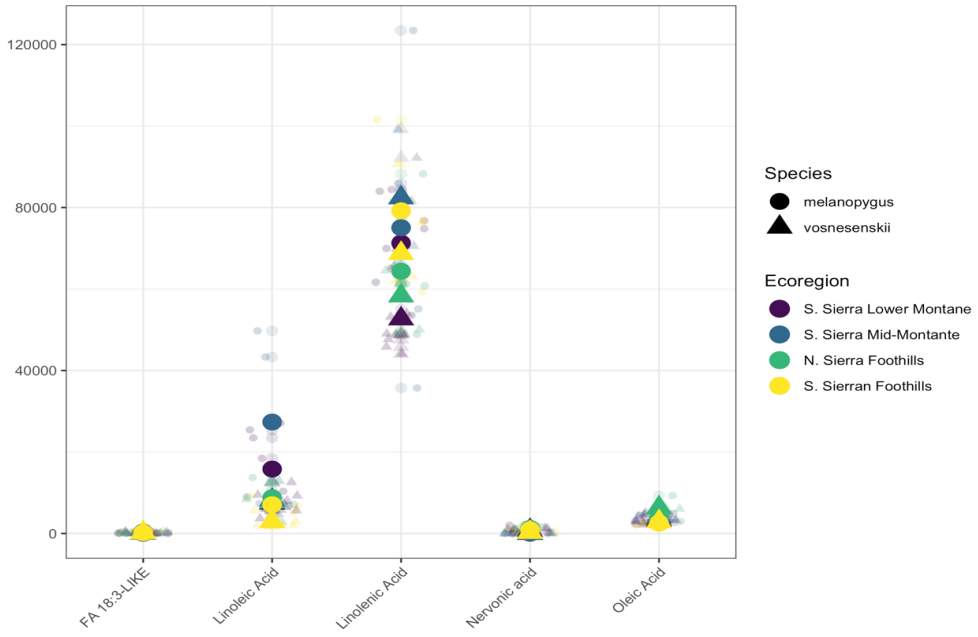

Figure 3.5: Relative abundance of fatty acids in pollen. Oleic acid was higher in pollen collected from the Northern Sierra Foothills but no other fatty acids exhibited differences between species or ecoregions. Individual points are jittered to more easily visualize overlapping points (width +\- 2, height +\- 0.3).

Discussion

Pollinators use vision and chemoreception to perceive cues from floral resources at multiple scales, including floral resource identity, and corresponding traits like flower

color (Fenster et al., 2004) and nutritional quality (Kitaoka & Nieh, 2009; Ruedenauer et al., 2016), and floral resource availability (Thomson, 1981; Fowler et al., 2016). These multi-scale, plant-pollinator, interactions ultimately drive floral visitation. Recent laboratory choice assays have demonstrated that specific micronutrients in pollen (Kitaoka & Nieh, 2009; Ruedenauer et al., 2016), macronutrient ratios in pollen (Vaudo et al., 2016; Vaudo et al., 2018; Kraus et al., 2018), and pollen content (monofloral vs. polyfloral) (Eckhardt et al., 2014) influence floral resource collection, and subsequently brood development (Moermann et al., 2017; Watrous et al., 2019), in bumble bees. Our understanding of how the scale of nutrients contributes to floral resource visitation where these other factors are also present is however limited. This study is the first to explore nutrients collected from pollen in heterogeneous floral resource environments.

We collected pollen loads from two generalist species of bumble bees in four distinct ecoregions so that we could explore relationships between pollen nutrients and floral visitation by wild bumble bee species. *B. melanopygus* species peak foraging period occurs approximately one month before *B. vosnesenskii*. As a result, these species are often exposed to different floral resources temporally and thus display unique floral visitation patterns across their respective foraging periods (Cole et al., 2020). These species do share some overlap in foraging phenology and can share floral niches during this overlapping period (Williams et al., 2014; Cole et al., 2020), though they are at different colony developmental stages, which may influence foraging preferences. In our study, individuals were collected from shared meadows on the same day, and thus had access to similar floral resources. This allowed us to begin to examine species-specific

differences in floral preferences. Generalist pollinators are also limited by the floral community in which they forage, and they are only able to exhibit preferences when multiple choices are available. California is a region of high plant biodiversity and endemism, and plant communities vary dramatically across ecoregions. By sampling across ecoregions where both of these species are present, we could compare pollen collection in different floral resource environments. Floral resource collection may be a function of preference for plant traits or because of easier accessibility to particular plants, even if these plants are not necessarily preferred in a binary choice assay or are more beneficial (Heinrich, 2004). Our findings provide further evidence suggesting that pollen nutrients might impact floral visitation (Somme et al., 2015; Vaudo et al. 2016).

When we compared nutrient contents of pollen loads collected by the two focal species foraging from similar ecoregions, we found species-specific differences in metabolite richness. Specifically, *B. vosnesenskii* often collected a higher number of pollen metabolites than *B. melanopygus*. We also found species-specific differences when we performed focal analyses of similarity for amino acids, sterols, and fatty acids. However, Shannon diversity of pollen metabolites did not differ between species. The finding that metabolite richness and composition differed between species from the same ecoregions suggests that species may be flexible in the specific nutrients that they collect and potentially exhibit preferences for specific nutrients, based on what is available in the floral resources in their foraging environment. Bumble bees may collect core nutritional components at similar relative quantities (Moerman et al., 2017; Kriesell et al., 2017), but the specific compounds can be different depending on the foraging environment

(Saifuddin and Jha, 2014). Collection of additional compounds, which would change overall richness, will not change Shannon diversity if they are collected in small, relative quantities. This would explain the differences in richness and similarity between species, but similar Shannon diversity indices.

Richness and diversity of metabolites in pollen did not vary significantly between ecoregions. Overall similarity in composition of pollen metabolites, which considers metabolite identity, as well as abundance of individual amino acid, sterol, and fatty acid metabolites, did differ between ecoregions. Thus, there were the same relative amounts of metabolites in pollen to potentially discriminate between, but there were different kinds of metabolites present in pollen, depending on the floral resource environments. These findings provide evidence that bumble bees have different nutritional choices in different floral resource environments, and this may subsequently impact floral visitation. Our sampling design did not allow us to disentangle whether nutrients in plant pollen were the driver of pollen collection, but we did observe both species- and ecoregion-level differences that provides support for our hypothesis that nutrients in pollen influence pollen collection in heterogeneous floral resource environments where choices are possible.

Irrespective of what drives differences in metabolite contents of pollen, there are known down-stream effects of pollen quality on bumble bee colony development (Watrous et al., 2019) and health (Moermann et al., 2017), which can thus impact bumble bee populations in these regions where pollen metabolite composition varies. Bees preferentially forage for specific nutritional profiles, like specific amino acids (Kriesell et

al., 2016) or sterols (Vanderplanck et al., 2020) and may obtain nutrient targets via pollen collection from multiple plant species (Kriesel et al., 2017). There are ten amino acids that bees need to collect from floral resources because they cannot produce them de novo and are thus referred to as essential amino acids (de Groot, 1957). These are likely to be essential in bumble bees as well (Genissel et al., 2002), which suggests that bumble bees also must exclusively obtain these amino acids from plants. Less is known about the specific sterols that bees interact with, though they are necessary steroid precursors of ecydsteroid, which is a core hormone involved in insect pupation and is thus necessary for offspring to develop into adults. Similar to the essential amino acids, sterols are exclusively collected from floral resources by bees (Svoboda et al. 1978; Behmer, 2009). Some fatty acids can be produced endogenously, though omega-3 and 6, which have both been implicated in honey bee cognition, must be collected from floral resources (Manning, 2007).

We found contrasting patterns in amino acid quantity in pollen collected by different species in different ecoregions. For example, quantity of arginine and threonine, two essential amino acids, was different depending on bumble bee species and ecoregion but valine, another essential amino acid, was present at similar levels in pollen regardless of species or ecoregion. This is inconsistent with a previous study that compared amino acid concentrations in pollen collected by different species that did not find differences in relative amounts of amino acids collected. This study was performed in one habitat which may have had fewer floral resources for bees to collect pollen from and thus fewer choices (Kriessel et al., 2017). Bumble bees are known to adjust collection of protein to

balance nutrient requirements in response to changes in protein and carbohydrate stores in the colony (Kitaoka & Nieh, 2009; Hendriksma et al., 2019). It is thus reasonable to expect that amino acid collection could be flexible depending on what amino acids are available in the floral resource environment.

We found relatively high amounts of the sterols campesterol, episterol, and stigmasterol in pollen and the variation in relative quantity of each of these sterols was dependent on species and ecoregion. This may be because sterol content varies substantially between plant species (Vanderplank et al., 2014) and that different bees may preferentially collect different phytosterols to address nutrient requirements of their offspring. Campesterol and sitosterol were previously found to be abundant in pollen collected by bumble bees and were important for bumble bee colony development (Moermann et al., 2017).

We also found relatively high quantities of other amino acids, sterols, and fatty acids in pollen that did not vary between species or ecoregion. This included the fatty acid, linolenic (omega-3) acid, which had the highest relative amount compared to other fatty acids, followed by linoleic (omega-6) acid. These are both essential fatty acids found in pollen (Manning, 2007). Deficiencies in omega-3 fatty acids have been shown to impair learning and cognition in honey bees (Arien et al., 2015), and diets high in omega-6:3 ratios have been shown to negatively impact learning (Arien et al., 2018), both of which are important for bees to effectively navigate floral resource environments. Similarly, the sterols fucosterol-like and lupeol, as well as the non-essential amino acid glutamine, were found at relatively high amounts compared to other sterols and amino

acids in pollen but did not vary based on ecoregion or species. The presence of these compounds in pollen across both species and ecoregions may be a result of their importance to colony development and health, or because they are simply present in more floral resources compared to other compounds.

When foraging for resources, bumble bees must navigate floral resource environments to satisfy the nutritional requirements of many individuals with very diverse nutritional requirements (Lihoreau et al., 2018). Though theoretical frameworks, such as nutritional geometry, have recently been developed for social insects in order to understand how social animals satisfy their nutritional requirements (Lihoreau et al., 2015), there is currently a dearth of empirical data to test these theoretical models and test whether nutrients mediate interactions between bees and floral resources. Our study provides a comprehensive overview of how the collection of multiple classes of pollen nutrients vary based on species and habitat. These results provide an overview of important nutrient classes in pollen. These nutrients can thus provide important candidate nutrients that may play an important role in floral visitation, though this needs to be explicitly tested in future studies.

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Chapter Four: A contemporary survey of bumble bee diversity across the state of California

Abstract

Bumble bees (genus *Bombus*) are important pollinators with more than 260 species found worldwide, many of which are in decline. Twenty-five species occur in California with the highest species abundance and diversity found in coastal, northern, and montane regions. No recent studies have examined California bumble bee diversity across large spatial scales to identify regional differences in their composition. We collected 1740 bumble bee individuals, representing 17 species from 17 sites (~100 bees per site) in California using an assemblage monitoring protocol. This protocol is intended to provide an accurate estimate of relative abundance of more common species without negatively impacting populations through overcollection. Our sites were spread across six ecoregions, with an emphasis on those that historically housed greater bumble bee diversity. We compared bumble bee composition among these sites to provide a snapshot of California bumble bee biodiversity in a single year. Species diversity was highest at two sites located in the Sierra Nevada and the Central Basin, respectively, and average site species richness was 4.5 +/- SE 0.4. *Bombus caliginosus* was the only rare or threatened species observed during sampling. Our study sheds light on the current status of bumble bee diversity in California and identifies some areas where greater sampling effort and conservation action should be focused in the future.

Introduction

The state of California encompasses the majority of the Mediterranean-climate California Floristic Province, one of the world's top 25 biodiversity hotspots (Howell, 1957; Mittermeier *et al*., 1999). Given its high levels of biodiversity and species endemism, the state is a critical target of global conservation efforts (Myers *et al*., 2000). California is also among the most impacted by global changes such as rapid urbanization and development, agricultural intensification, and climate change (CDFW, 2015), which are threatening biodiversity throughout the state. Among its diverse and threatened taxa, California is home to 25 bumble bee species (Hymenoptera: Apidae, *Bombus* Latrielle), which equates to approximately 50% of all North American species (Williams *et al*., 2014) and ~10% of those worldwide (Williams, 1998). The genus *Bombus* is comprised of approximately 260 species globally. It includes the social subgenera, which have a reproductive caste that includes males and females and a non-reproductive female worker caste, as well as the socially parasitic subgenus *Psithyrus* Lepeletier, which only produces reproductive males and females. Six California bumble bee species are considered of conservation concern within the state by the California Department of Fish and Wildlife (CDFW): *B. caliginosus* Frison, *B. crotchii* Cresson, *B. franklini* Frison, *B. morrisoni* Cresson, *B. occidentalis* Greene, *and B. suckleyi* Greene (CNDDB, 2020). These species are also considered threatened across their entire ranges by the International Union for the Conservation of Nature (IUCN), where three are assessed as vulnerable (*B. caliginosus, B. morrisoni, B. occidentalis*), one as endangered (B. crotchii), and two as critically endangered (*B. franklini, B. suckleyi*) (Hatfield *et al*., 2014). Bumble bees as a

group are experiencing substantial declines worldwide (Goulson *et al*., 2008; Williams & Osborne, 2009), with ~⅓ of species in North America considered in decline by the IUCN (Hatfield *et al*., 2014; Arbetman *et al*., 2017). Therefore, understanding bumble bee diversity patterns in regions of high conservation concern is particularly valuable.

Efforts to characterize the current status of bumble bee populations in California are necessary for establishing baseline information about relative abundance, continuing to develop more refined range maps, and ultimately conserving this pollinator group in the context of the state's rapidly changing landscapes and climate. Bumble bees are found throughout most of the state; however, they tend to be most abundant and speciose in northern, coastal, and montane areas, and much less so in the Central Valley and Southern California, especially at lower-elevation sites in the Mojave and Sonoran Deserts (Thorp *et al*., 1983). An analysis of bumble bee biodiversity was performed statewide in the early 1980s by Thorp *et al*. (1983), but there have been no other statewide studies since. More contemporary studies have been performed in targeted areas of the state, including parts of the Sierra Nevada Mountains (Hatfield & LeBuhn, 2007; Loffland *et al*., 2017) and urban areas along the coast (McFrederick & LeBuhn, 2006; Schochet *et al*., 2016). Additional studies have examined entire bee communities (including bumble bees) in specific areas in the state, such as the decades-long bee monitoring effort at Pinnacles National Park (Meiners *et al*., 2019).

Several of California's threatened bumble bee species began to decline precipitously beginning in the late 1990s and early 2000s (Thorp, 2005; Koch *et al*. 2009; Cameron *et al*., 2011b; Hatfield *et al*., 2014; Graves *et al*., 2020). However, there have

been no contemporary, large-scale efforts to assess bumble bee species assemblage composition in the state since these declines began. To begin to fill this important information gap, we investigated the composition of the bumble bee assemblage across California during the 2019 nesting season. We collected bees from a set of 17 sites distributed across the state, with an emphasis on the Coast Range, Klamath, and Sierra Nevada ecoregions (Figure 1). These areas historically harbored the most diverse and abundant bumble bee communities (Thorp *et al*., 1983). We employed an assemblage monitoring framework modeled after Strange & Tripodi (2018), who assessed bumble bees across most of the United States (albeit not in California). Use of this protocol allowed us to maximize our ability to assess general trends in bumble bee community composition across our sites, while minimizing negative impacts on bumble bee populations through overcollection. This approach is highly informative for inferring bumble bee community composition at individual sites at particular time points (Strange & Tripodi, 2018).

Many monitoring protocols tend to prioritize rare and threatened species in order to assess their conservation statuses and inform efforts to conserve them. However, monitoring is also needed for more common species so that they can also be targeted for conservation efforts if they begin to decline, in order to prevent substantial declines in the future and avoid extinction debt (Kuussaari *et al*., 2009). Our one-year study provides general insights into how bumble bee communities differ between some of the more speciose regions of California. We also provide additional evidence about regions that

contain the highest biodiversity and rarest species, which might be prioritized for conservation efforts.

Methods

Site selection and sampling

Collection sites were selected based on their accessibility (by vehicle and/or by foot), their approval through permits, and their distribution across the state and ecoregions, with an emphasis on the coastal and montane regions that are most speciose (Thorp *et al.,* 1983). All sites were >12 km apart. We assigned all of our sampling sites to their EPA Level III Ecoregion classifications (Griffith *et al.,* 2016) in accordance with practices of the CDFW and U.S. Fish and Wildlife Service (USFWS; Fig. 4.1). We collected all specimens during spring and summer 2019 (May-August). We aimed to collect approximately 100 bees at each site over 1-3 consecutive collection days and between the hours of 1000-1800, following methods of Strange & Tripodi (2018). All bees were collected on days where there was no rain or high winds. Individuals were hand-netted at random, with the exception that we did not collect queens (usually distinguishable by their size and/or color pattern, relative to worker-caste females) of any non-parasitic species in order to minimize negative impacts on populations. We collected bees primarily from flowers, and when possible, we recorded associated plant species data. Bees were collected into 70% ethanol and stored at -80°C to preserve tissue for future analyses.

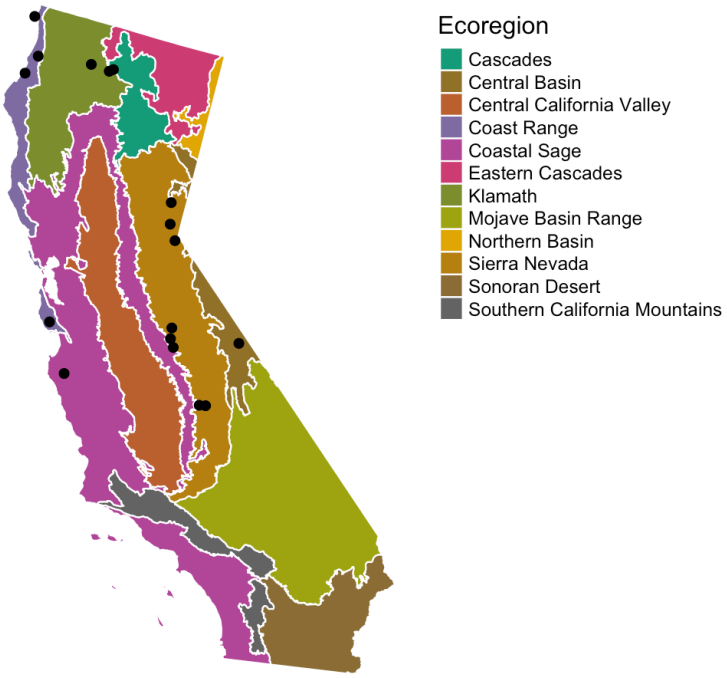

Figure 4.1: Map of the seventeen sites sampled across six of the thirteen Level III Ecoregions in California.

Bumble bee identification

Following collection, all bees were removed from ethanol (with a single leg retained in ethanol as a DNA voucher), rinsed with ethanol, dried, then pinned. Bees were identified to species following diagnostic characteristics in Thorp *et al*. (1983), Stephen (1957), and Williams *et al*. (2014), and were retained by the Woodard Lab at UC Riverside. We refer to some species listed in Thorp et al. (1983) with updated taxonomic nomenclature following Williams *et al*. (2014): *B. californicus* now as *B. fervidus*, *B. sonorus* as *B. pensylvanicus*, *B. fernaldae* as *B. (Psithyrus) flavidus, B. edwardsii* as *B. melanopygus*, and *B. bifarius* species complex as *B. bifarius* (Ghisbain *et al*., 2020). Differentiation between *B. vosnesenskii* and *B. caliginosus*, two easily mistaken species, was handled as follows. *B. caliginosus* is only known to occur in the coast ranges, according to Thorp et al. (1983) and the Discover Life website. This is further validated by Williams et al. (2014), who do not describe *B. caliginosus* as occurring east of the Central Valley. In the sites where they do putatively co-occur, we used morphological characteristics from Williams et al. (2014). Specifically, in *B. vosnesenskii*, the malar space is not longer than wide; T4 is completely yellow; S3-4 has only black hairs; and there are many large pits on the lower central area of the clypeus. In *B. caliginosus*, the malar space is longer than wide; the leading edge of T4 has many black hairs medially; S3-4 has yellow hairs; and there are only small or only a few large pits on the lower central area of the clypeus. We found these morphological characteristics to be consistent with previously described species locations; no bees resembling *B. caliginosus* were detected outside of our Coast Range sites. Bee species identities, floral associations, and

associated data are available through Dryad (will be deposited upon publication). All specimen data have been deposited in the CDFW's California Natural Diversity Database.

Statistical methods: All statistical analyses were performed using R version 4.0.2 (R Core Team, 2020). We estimated study-wide relative abundance for each species by dividing the total number of each species across all sampling sites by the total number of bees collected across all sampling sites. To estimate species richness and Shannon (H') diversity for each site and ecoregion, we generated sample-size-based rarefaction and extrapolation curves with 95% confidence intervals using the iNEXT package (v. 2.0.2; Hsieh *et al*., 2016).

To visualize similarity in bee assemblage between each site, we used non-metric multidimensional scaling analyses (NMDS) with a Bray-Curtis dissimilarity index (Minchin, 1987; Warton *et al*., 2012). This was followed by an analysis of similarity (ANOSIM) in the Vegan package (v. 2.5-6; Oksanen *et al*., 2019) to test whether there were differences in species composition between ecoregions. The ANOSIM compared the mean distance between sites within an ecoregion to the mean distance among sites between ecoregions based on species clustering patterns (Clarke, 1993). We performed the NMDS and ANOSIM on 1) abundance of every species at each site, and 2) presence-absence data at each site, in order to explore whether similarities or differences in bee assemblages between sites were due to the abundance of shared species or species identity (Williams, 2011).

To contextualize our species richness results with species historically found in the sampled ecoregions, we also obtained historical bumble bee specimen data from 35 public and private collections, assembled by L.L. Richardson (Williams *et al.,* 2014; Richardson *et al.,* 2020; Table 2). This database includes the majority of bumble bee specimens used by Thorp *et al*. (1983), among other museum collections, and is the best available digital repository of historical records in California (See Appendix for complete list of collections). We did not directly compare the current survey data to historical records quantitatively because of major differences in sampling approach and coverage. Instead, this comparison is intended to provide insight into differentiating between species that are still relatively likely to be collected at sites, across historical and current collection events, versus species that are either present but relatively rare or are no longer present at a site.

Results

We collected a total of 1740 bumble bees across 17 collection sites (mean number of bees per site = 102 +/- SE 2; range = 84-110) in six different ecoregions (Figure 4.1). Our resulting data set includes 17 different *Bombus* species, representing 68% of bumble bee species known to inhabit California historically and 34% of the approximately 50 U.S. bumble bee species. 28% of all bees collected were males and the remainder were females of the worker caste, or females of parasitic species.

Species	Sites	Ecoregions	Total Relative Abundance %
B. vosnesenskii (Radoszkowski)	16	6	57
B. melanopygus (Nylander)	9	4	8
B. bifarius (Cresson)	6	3	7
B. flavifrons (Cresson)	5	3	7
B. rufocinctus (Cresson)	11	5	4
B. mixtus (Cresson)	4	2	4
B. caliginosus (Frison)	5	2	2
B. huntii (Greene)	1	1	2
B. vandykei (Frison)	5	2	1
*B. flavidus** (Eversmann)	4	4	1
B. fervidus (Fabricius)	3	3	1
B. insularis* (Smith)	3	2	1
B. kirbiellus (Curtis)	1	1	1
B. sylvicola (Kirby)	1	1	3
B. centralis (Cresson)	1	1	0.3
B. griseocollis (De Geer)	1	1	0.2
B. appositus (Cresson)	1	1	0.1

Table 4.1. Summary table for species
*Indicates subgenus *Psithyrus*

Common and rare species in the dataset

The most commonly collected species was *B. vosnesenskii*, which represented more than half (~57%) of all collected bees. This species was collected in all six ecoregions we sampled, and at all of our sites except for one in the Sierra Nevada ecoregion (Site NS2). With respect to relative abundance, this species was followed only distantly by *B. melanopygus,* which represented 8% of all bees collected and was collected at nine sites across four ecoregions (Table 4.1).

The rarest species in our data set were *B. appositus* (n = 2 specimens collected), *B. griseocollis* (n = 3), and *B. centralis* (n = 5). Each of these species was collected at only a single site, and each from a unique ecoregion (Sierra Nevada, Central Basin, and Coast Range, respectively). Four species were collected exclusively from one site in the Central Basin: the high-elevation species *B. kirbiellus* (n = 12), *B. centralis* (n = 5), *B. huntii* (n = 28), and *B. sylvicola* (n = 48). We detected two species in the socially parasitic subgenus *Psithyrus*: *B. insularis* was collected at three of our sites in the Sierra Nevada and Cascades ecoregions (n = 21 bees collected across all sites) and *B. flavidus* was collected at four of our sites in the Sierra Nevada, Cascades, Klamath and Coast Range ecoregions (n = 23 bees collected across all sites).

All of the species we collected (n = 17) have historically been reported in the same ecoregions where we collected them (Table 4.2). However, many of these species were collected in only a subset of the ecoregions where they have been historically found. For example, *B. caliginosus*, the only imperiled species we observed, was collected at five sites (n = 36 bees). Although this species was historically present in four of the

ecoregions where we sampled, we only collected it in two ecoregions, Coastal Sage and Coast Range (Table 4.2).

Ecoregion	# Sites (2019)	Total species Richness (2019)	Average Species Richness (2019)	Historical Species Richness	Species
Cascades	1	6	6	20	appositus, **bifarius**, caliginosus*, centralis, fervidus**, flavidus**, flavifrons, franklini*, griseocollis, **insularis,** melanopygus, mixtus, morrisoni, nevadensis, occidentalis*, **rufocinctus,** suckleyi*, sylvicola, **vandykei, vosnesenskii**
Central Basin	1	7	7	18	appositus, bifarius, **centralis**, crotchii*, **fervidus**, flavifrons, griseocollis, **huntii**, insularis, **kirbiellus**, melanopygus, mixtus, morrisoni*, nevadensis, pensylvanicus, rufocinctus**, sylvicola, vosnesenskii**
Coastal Sage	1	4	4	21	appositus, bifarius, **caliginosus***, centralis, crotchii, **fervidus**, flavidus, flavifrons, griseocollis, huntii, insularis, **melanopygus**, mixtus, nevadensis, occidentalis*, pensylvanicus, rufocinctus, sitkensis, sylvicola, vandykei, **vosnesenskii**
Coast Range	4	9	4	19	bifarius, **caliginosus***, centralis, crotchii*, **fervidus, flavidus**, flavifrons, **griseocollis,** huntii, insularis, **melanopygus**, **mixtus**, occidentalis*, pensylvanicus, **rufocinctus**, sitkensis, vandykei, **vosnesenskii**
Klamath	2	6	4	21	**bifarius**, caliginosus, crotchii*, fervidus**, flavidus**, **flavifrons**, franklini*, insularis, griseocollis, huntii, **melanopygus,** mixtus, morrisoni*, occidentalis*, **rufocinctus**, sitkensis, suckleyi*, sylvicola, vandykei, **vosnesenskii**
Sierra Nevada	8	10	5	22	appositus, **bifarius**, centralis, crotchii*, fervidus, **flavidus, flavifrons**, griseocollis, huntii, **insularis**, kirbiellus, **melanopygus, mixtus**, morrisoni*, nevadensis, occidentalis*, pensylvanicus, **rufocinctus,** sitkensis, sylvicola, **vandykei, vosnesenskii**

Table 4.2: Contemporary (2019) and historical species richness and estimated species richness by Ecoregion
Number of bees collected per site averaged 102 +/- 1.5 SE (Range: 84 – 110)
Bold indicates species that were found in 2019 and historically
Un-bolded indicates species only found historically
*Considered imperiled by CDFW

Bumble bee species richness

Estimated species richness was comparable to observed species richness for nearly all of the sites (15 of 17). For 15 sites, our estimates of species richness reached asymptotes and extrapolation did not predict that we would observe more species with greater sampling effort (Fig. 4.2). The two exceptions were one site in the Central Basin (8 species estimated; 7 species observed) and one site in the Klamath Mountains (6 species estimated; 4 observed). A site in the Central Basin, and another in the Sierra Nevada, had the highest α diversity, whereas a different site in the Sierra Nevada, as well as a site in the Coast Range had the lowest α diversity (Fig. 4.2). At the ecoregion level, average species diversity (i.e., average α diversity across all sites in an ecoregion) was highest in the Central Basin (Mean Richness = 7 +/- SE NA; Mean H' = 1.6 +/-SE NA) and lowest in the Klamath Mountains (Mean Richness = 4 +/- SE 0; Mean H' = 0.6 +/- SE 0.1) though Central Basin was only informed by a single site (Table 4.2). Gamma diversity (ecoregion level diversity) was highest in the Sierra Nevada (Richness = 10; H' = 4.6) and the Coast Range (Richness = 9; H' = 3.3), although some sites in these ecoregions had low alpha diversity relative to gamma diversity. Gamma diversity was lowest in the Coastal Sage ecoregion (Richness = 4, H' = 2.7) (Table 4.2).

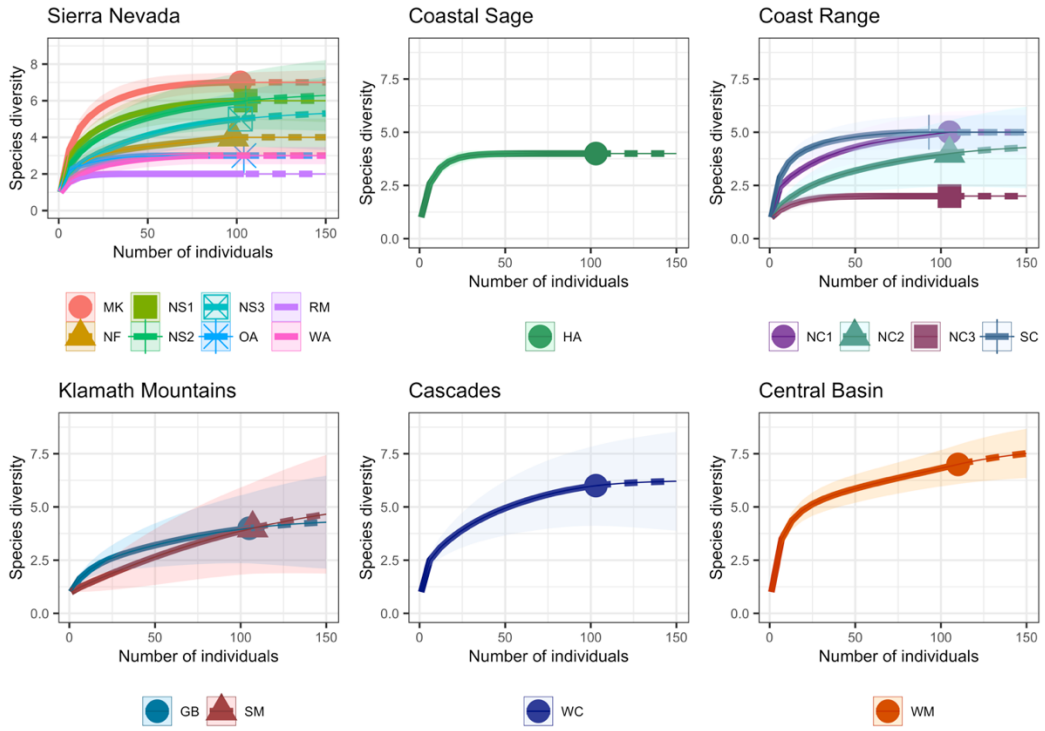

Figure 4.2: Rarefaction curves generated from estimated species richness for each site according to Level III Ecoregion. Extrapolation estimates are based on sampling 150 specimens.

Community composition

There were no differences in community composition among ecoregions when we considered both species abundance and identity (Figure 4.3A; $R^2 = 0.983$; stress = 0.13; ANOSIM Ecoregion: $R = 0.0611$, $p = 0.34$). However, differences between ecoregions were detected when we considered only species identity (Figure 4.3B; $R^2 = 0.985$; stress = 0.121; ANOSIM Ecoregion: $R = 0.3909$, $p = 0.0078$). We found two distinct ecoregion groupings: Group One included the higher-elevation, mountainous ecoregions Klamath, the Sierra Nevada, and the Cascades, whereas Group Two included the lower-elevation ecoregions Coastal Sage, Coast Range, and the Central Basin (ANOSIM Group: $R = 0.5747$, $p < 0.001$). There were no significant differences in composition between the ecoregions within Group One (ANOSIM Ecoregion Group One: $R = -0.04377$, $p = 0.5669$) or Group Two (ANOSIM Ecoregion Group Two: $R = 0.3704$, $p = 0.3333$).

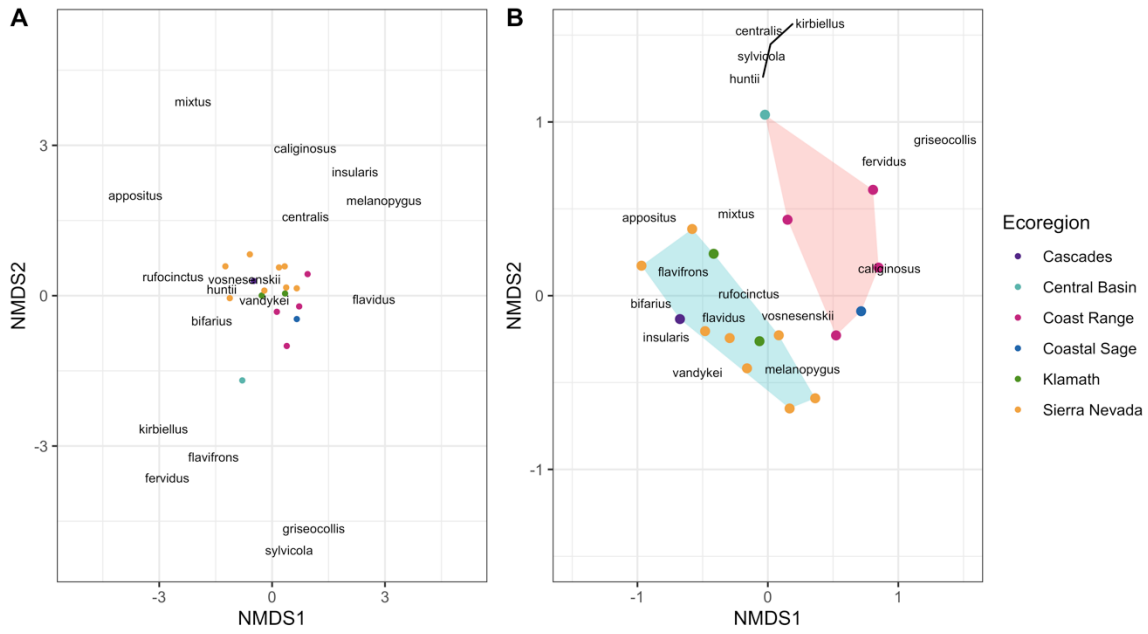

Figure 4.3: Non-metric multidimensional scaling of study sites using A) Species abundance and identity and B) Presence/Absence of species. Ordinations were based on Bray-Curtis dissimilarity. Circles represent sites, which are clustered relative to species shared between sites (indicated with species name). Lines in B indicate shared location for *B. centralis*, *B. huntii*, *B. kirbiellus*, and *B. sylvicola*; species names are offset for better visualization.

Discussion

Our study provides the first broad overview of California bumble bee species diversity in nearly 40 years (Thorp *et al*., 1983). During this period, California has undergone considerable landscape-level changes due to agricultural intensification, urbanization, and climate change (CDOC, 2021), all of which affect bumble bee habitat, making our study particularly timely. We sampled approximately 100 bees from each of our 17 sites, which were spread across six ecoregions in California. Our results thus provide a modern overview of bumble bee richness throughout the state and can be used as a contemporary baseline for future monitoring efforts.

The widespread distribution of four dominant species likely explains why community composition did not differ among ecoregions when we performed this analysis using species abundance metrics. *Bombus vosnesenskii* is highly abundant throughout California and was the dominant species across all of our sites, which is consistent with previous studies of statewide patterns by Thorp *et al*. (1983) and in the Sierra Nevada by Loffland *et al*. (2017). This species represented more than half of the specimens in our study and was present at all of our sites, with the exception of one site in the Sierra Nevada. Similarly, *B. melanopygus, B. bifarius*, and *B. flavifrons,* which had the three next highest relative abundances after *B. vosnesenskii*, were found in more than half of the ecoregions where we sampled. The rarer species that turned over among sites and regions did not influence abundance-weighted metrics of community similarity. When we only considered species presence or absence, however, we revealed significant differences in composition between ecoregions. Thus, rarer species we collected

contribute most to the beta diversity among ecoregions. Species turnover, or beta diversity, is the proportion of species composition that changes between sites. Similar patterns have been found in other analyses of spatial beta diversity in bees (Winfree et al. 2018) as well as in other taxa (Cardinale et al. 2011; Isbell et al., 2011). When considered in terms of presence-absence, higher elevation sites appeared to have greater species similarity compared to the lower elevation sites. The differences between these two groups may be due to substantial variation between these two groups, rather than similarity within the lower elevation group. This is exemplified by the Central Basin having four unique species, even though there were no significant differences between the Central Basin and coastal ecoregions. Moreover, we only sampled at one site in this region, and species turnover may be detected with greater sampling. Sampling at more sites could potentially yield higher ecoregion level richness and thus more pronounced differences in species composition compared to the other ecoregions.

We sampled a fairly consistent number of individuals at each of our sites (84-110), although we had an uneven representation of sites in each ecoregion (range of 1-8 sites per ecoregion). Thus, patterns of richness were different based on whether average site diversity for each ecoregion (α diversity) or ecoregion level diversity (γ diversity) was considered. In the Sierra Nevada and Coast Range, where we collected at more sites, we detected higher gamma diversity but lower average site diversity compared to other ecoregions. For example, the site with the highest richness and the site with the lowest richness were both in the Sierra Nevada. The Central Basin and Cascades, in contrast, had the highest average site diversity (average α diversity). This suggests beta diversity

contributes importantly to total regional diversity of bumble bees and that collections at additional sites in the Cascades and Central is needed to understand regional diversity patterns.

Based on our species accumulation analysis, estimated species richness was equivalent to observed species richness at most of our sites, suggesting that our sampling protocol generally represented the bumble bee assemblage that was present at our sites during the time of sampling. This mirrors what was found by Strange and Tripodi (2018) in their assessment of bumble bee communities across the United States, though they did not sample in California. Two exceptions to this include the site in the Central Basin, and one site in the Klamath Mountains, where estimated richness exceeded observed. These ecoregions historically harbored high numbers of bumble bee species, in addition to hosting species that are rare in other ecoregions in California. For example, *B. kirbiellus* was historically present at high elevations in the Central Basin and Sierra Nevada, but we only observed it in the Central Basin. *Bombus suckleyi* has historically only been observed in the Cascades and in the Klamath Mountains (Thorp *et al*., 1983; Table 2).

For the species that were found historically in a region, that we did not collect in our study, it is unclear whether these species were not collected because (i) they are rare and require greater sampling to detect, (ii) they require sampling at a different time point in the season, or (iii) they are no longer present in an area. Thus, we warrant caution in the interpretation of our comparison between historical and contemporaneous (our) data. More intensive sampling, including at multiple points across the bumble bee flight season, are required to differentiate between different explanatory scenarios. However,

the eight species that we did not collect in our study include five species that are considered to be of conservation concern by CDFW and the IUCN (*B. occidentalis*, *B. suckleyi*, *B. franklini, B. crotchii*, and *B. morrisoni*). Thus, their increasing rarity may partially explain why we did not collect them in our study. *Bombus occidentalis* was historically widespread across the state (Thorp *et al*., 1983), but is now restricted to high meadows and coastal environments in the northern most part of the state (CDFW, 2019; Graves *et al*., 2020). This species appears to still be sporadically present in the Sierra Nevada (Hatfield & LeBuhn, 2007; Cole *et al*., 2020), and the Northern Coast Range (Graves *et al*., 2020), although we did not observe it in our sampling in either of these ecoregions, which is consistent with another more recent study in the Sierra Nevada (Loffland *et al*., 2017). The documented decline of *B. occidentalis* began in the mid-1990s, in the most western parts of its range, including in California (Cameron *et al*., 2011a; Graves *et al*., 2020). The decline of *B. occidentalis* is also a driving factor in the decline of *B. suckleyi*, a socially parasitic species that depends on *B. occidentalis* as its host (Thorp *et al*., 1983; Lhomme & Hines, 2019). Species at higher trophic levels, including cleptoparasites, are more vulnerable to decline than those at lower trophic levels (Klein *et al*., 2006; de la Mora *et al*., 2020). *Bombus franklini* was last observed in California in 1998 and in Oregon in 2006, and is considered Critically Endangered by the IUCN (CDFW, 2019). This species has the smallest known range of any bumble bee species and was historically restricted to southern Oregon and Northern California (Plowright & Stephen, 1980; Thorp 2005; Williams *et al*., 2014).

We may not have collected *B. crotchii* and *B. morrisoni*, as well as the remaining three species that are not currently considered to be threatened (*B. nevadensis*, *B. sitkensis*, and *B. pensylvanicus*), because of the timing and/or location of our sampling, rather than their extreme rarity. *B. crotchii* and *B. morrisoni* are both present in recent California observations reported to online community science databases (e.g., iNaturalist, Bumble Bee Watch). Bumble bee species differ phenologically, whereby peak abundance of workers occurs at different times throughout the foraging season depending on the species (Goulson, 2003; Williams *et al.,* 2014). In some ecoregions we sampled a preponderance of males, whose presence indicates the approach of or end of a flight season for a species (Goulson, 2003) in some of these ecoregions. We therefore may have missed the peak abundance of some of the species we did not collect but were expecting to find. Moreover, although we sampled in ecoregions where all of these species were historically present (Thorp et al. 1983; Cole *et al.,* 2020, Table 2), we had a limited number of sites in the ecoregions where *B. nevadensis* (Thorp *et al.,* 1983; Cole *et al.*, 2020) and *B. morrisoni* have previously been collected (Thorp *et al.,* 1983; CDFW, 2019; Graves *et al.,* 2020). Similarly, the current range of *B. crotchii* and *B. pensylvanicus*, which were previously abundant throughout California, appears to now be restricted to xeric and coastal sites in Southern California (Thorp *et al.*, 1983; Schweitzer *et al.*, 2012; Richardson & Woodard, unpublished data) and the Central Valley, where we did not sample.

Bombus caliginosus was the only threatened species we observed during our sampling. Although this species is considered threatened in the state by CDFW, our data

suggest that it is potentially less rare in the coastal areas where it occurs, given that this species comprised up to 16.5% of the community in some sites where we sampled. *Bombus caliginosus* is easily mistaken for *B. vosnesenskii* (Stephen, 1957; Thomson, 2016), making differentiating between the two species in coastal regions challenging. We propose that, specifically in the coastal areas of California where their distributions overlap, the occurrence of *B. caliginosus* might be underestimated because it is mistakenly identified as the vastly more common *B. vosnesenskii,* especially in non-destructive sampling studies.

Our study has several limitations that need to be considered when drawing conclusions from our results. First, as discussed above, we sampled an uneven number of sites in each ecoregion, which prevented us from explicitly estimating species turnover between sites. Second, our sampling protocol specifically targeted more abundant species and may not have been sensitive to turnover of rarer taxa, which are often missed with standardized efforts, as opposed to targeted efforts to inventory rare species. As a result, we may have collected rare species in only a subset of the ecoregions where they were actually present. Finally, our collection was limited to a single season in one year, so it does not account for phenological turnover within the season, or for temporal variation in abundance among seasons or years. Each limitation likely prevented us from capturing full ecoregional diversity and important spatial variation. Nevertheless, our collections provide a critical snapshot of the relative abundance and diversity among sites across multiple ecoregions in California and can be used as a baseline for future monitoring efforts. Additionally, our study further validates the efficacy of Strange and Tripodi's

(2018) monitoring protocol, which can be employed to capture information about the bumble bee community at a site while minimizing harmful impacts on local populations from over-collecting.

Future bumble bee monitoring schemes should include repeated sampling periods across the flight season, as well as multiyear sampling, to capture phenological changes in bumble bee communities within ecoregions. More sites should be sampled and stratified among ecoregions, particularly the Cascades, Central Basin, and Coastal Sage, to increase the breadth of sampling in order to account for species turnover across sites, especially for rarer taxa. Sampling in additional regions of the state should also be included in future monitoring efforts to provide a more detailed picture of California bumble bee species distributions. These include, for example, the Southern California Mountains and the Central California Foothills. Similarly, the Central Valley and the southeastern part of the state, where bumble bees tend to be less abundant (Thorp *et al*., 1983), should also be targeted for extensive sampling to detect rarer species like *B. crotchii* and *B. pensylvanicus*. Results from more comprehensive sampling can then be rigorously compared to historical bumble bee distributions, and statistical analysis can be performed to shed light on the status and trends of bumble bee populations in the state.

Conservation efforts for California bumble bees are in progress at the state and national levels. *B. crotchii, B. franklini*, *B. occidentalis,* and *B. suckleyi* are considered to be imperiled by the CDFW (CDFW, 2018; CDFW, 2019) and *B. franklini* and *B. occidentalis* are currently under consideration for national listing by the United States Fish and Wildlife Services (USFWS) under the Endangered Species Act (ESA) (USFWS,

2019; Graves *et al*., 2020). Habitat loss, disease, competition for resources, and climate change may all be factors implicated in these species' declines (Colla *et al*., 2006; Williams *et al*., 2009; Cameron *et al*., 2011b; Hatfield *et al*., 2014; Kerr *et al*., 2015; Cameron *et al*., 2016). California is an extremely biodiverse state (Hamilton & Smyth, 2020), but it has undergone considerable recent changes that are implicated in widespread changes in biodiversity and are likely also increasing the number and strength of threats faced by bumble bees. Effective strategies for conserving California bumble bees should ideally consider how the different ecoregions across the state have been impacted by agriculture, urbanization, and climate change, as these in turn influence threatened bumble bee species in unique ways. For example, coastal ecoregions particularly in the south face the highest rates of urbanization, whereas Central Valley and Coastal Sage are most impacted by agricultural intensification (CDOC, 2021). Climate change is predicted to most significantly impact California's montane regions (Lenihan *et al*., 2003, 2008), which harbor considerable bumble bee abundance and diversity (Rappacciulo *et al*., 2014), by reducing snow accumulation, increasing temperatures (Cayan *et al*., 2008), and changing the plant communities that bumble bees rely on for floral resources.

Key barriers to successfully implementing bumble bee conservation actions in California include the lack of large-scale monitoring studies in the state, in addition to knowledge gaps in life history and drivers of species decline (Graves *et al*., 2020; USFWS, 2019). Overcoming these barriers and protecting California's bumble bee fauna is necessary for protecting the state from cascading negative impacts on agricultural and natural ecosystems (Macior, 1977; Thorp *et al*., 2002; Thorp, 2014; CDFA, 2018; Cole *et*

al., 2020). Conservation efforts that prioritize bumble bee diversity hotspots (e.g., areas with a high number of species per unit area), are also important for maximizing the number of species conserved. The assemblage monitoring framework we employed specifically provides insights into regions that should be prioritized for conservation based on this criterion. More broadly, our study highlights the need for greater monitoring of the diverse bumble bees of California in order to better understand the drivers of biodiversity and decline in this genus, and to more effectively manage bumble bee conservation.

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Conclusion

Feeding and foraging in bumble bees has been investigated in many different ways, which has produced substantial insights into how bumble bees navigate these two fundamental behaviors. In this dissertation, I built on these previous findings from other important scientists in the field by considering these behaviors at multiple scales. How do how organisms detect nutrients? How feeding is coordinated between individuals? What nutrients are present pollen collected by wild-caught bumble bee workers? What bumble bee species are present in different ecoregions throughout California.

In my first chapter, I found that B. impatiens have three taste-related gene family expansions and found that many taste genes are expressed in the mouthparts and tarsi. Future work exploring taste should thus focus on these two taste organs to better understand what compounds in the environment they play a role in detecting. In the second chapter, I found that most bumble bee workers feed brood, and that differences between workers appears to be determined by whether or not they collect pollen. Future work should thus explore whether there are physiological, genetic or cognitive (ie. differences in learning capacity) differences between these workers that may facilitate these behavioral differences. In Chapter Three, I identified nutrients present in bumble bee collected pollen, which is an important first step in exploring the potential role of nutrients in floral visitation by bumble bees. Finally, I characterized what bees are present in different ecoregions throughout California. I found 17 species across all ecoregions; *B. vosnesenskii* and *B. melanopygus* are the most common species throughout

the state, which may be linked to differences in their ability to effectively find food relative to less common species.

This work contributes to field of bumble bee biology by providing more knowledge as to how bumble bees interact with food resources. In the future, I plan to continue this work by exploring the role of taste in this interaction using functional genomics.