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Morphological Variance in Mouthparts and Foraging Behavior in Bumblebees

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Honors Thesis 2021

Colby College Department of Biology

Morphological Variance in Mouthparts and Foraging Behavior in Bumblebees

Honors Thesis

Presented to
The Faculty of the Department of Biology
Colby College

In partial fulfillment of the requirements for the
Degree of Bachelor of Arts with Honors

Ye Jin (Jane) Lee

Waterville, ME
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Reader: Devin O'Brien ____________________

Reader: Judy Stone JStone

CONTENTS

3. RESULTS AND DISCUSSION

ABSTRACT

Bumblebees (Hymenoptera: Apidae: Bombus) show an incredible degree of size variation within and between species. Individuals from the same hive may vary up to 10-fold in mass. This variation allows individuals to specialize in foraging on different flowers suited to their morphology. However, as different species have different foraging behaviors, their variation in mouthparts and scaling of mouthparts to body size may have been under different kinds of stabilizing selection as they adapted to collect nectar from flowering plants over evolutionary time. Here, we examined the scaling relationships between body size and mouthpart structures, and the variation in mouthpart shape between species to determine whether there is any relationship to foraging behavior. In addition, the wings were also analyzed as a reference trait to compare the trends seen in the mouthparts to. We sampled 8 bumblebee species throughout Maine, a region of high bumblebee species diversity, biogeographic variation, and diverse land-use histories. Variation in the size and shape of mouthparts was analyzed for each species using multivariate morphometric analyses to identify species and caste differences. Landmark based geometric morphometrics was used to study the wings. Our results indicate that there is significant correlation between morphological variance in mouthparts and foraging specialization in bumblebees. Specialist bumblebees such as *B.ternarius* and *B.terricola* have shallower scaling of tongue lengths to body size and lower variation in mouthpart shape than generalist species such as *B.bimaculatus*, *B.impatiens,* and *B.vagans*. Overall, these patterns support the hypothesis that different bumblebee species with different pollination strategies were under different stabilizing selection to result in different mouthpart morphology.

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Fini was always there for me in professor Dave's lab and also outside of it. She gave me guidance in the lab about the different techniques and concepts we were using, but also gave me advice and moral support for everything else that I was facing at or outside of Colby. She gave me the strengths to go through the ups and downs of research when everything worked out and when everything, especially the injections, failed to work.

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1. INTRODUCTION

1.1 Allometric Scaling

Allometry is the study of biological scaling, generally referring to the scaling relationship between different morphological structures of an organism with body size or between each other. Every aspect of an organism scales with body size in one way or another, and these scaling relationships are often consistent across a clade (Huxley and Tessier, 1936; O'Brien et al., 2019; Pebalon, 2014; Thompson, 1992).

Static allometry refers to the scaling relationship among individuals from the same population at a similar age or developmental stage (Cheverud, 1982; Cock, 1996; Pebalon et al., 2014). Examples of such scaling include the steep scaling of sexually selected structures relative to body size and the shallow scaling of many arthropod and vertebrate genitalia relative to body size (O'Brien et al., 2019; Shapiro and Porter, 1989; Pebalon 2013,2014).

Some sexually selected structures such as weapons of intrasexual competition and ornaments tend to be disproportionately large compared to body size (positive allometry). Examples include male dung beetle horns and harlequin beetle legs (O'Brien et al., 2019; Emlen, 2005; Kodric-Brown, 2006; Zeh, 1992). On the other hand, the lock-and-key hypothesis, or the one-size-fits-all hypothesis, states that structures such as genitalia of different individuals the same species are usually similar in size and structure regardless of body size due to stabilizing selection to allow for reproduction between all individuals in the same species (Cao et al., 2019; Shapiro and Porter, 1989; Pebalon, 2014; Eberhard, 2011). Another way stabilizing selection can result in a particular pattern of scaling can be seen in reproductive organs of flowering plants that interact with pollinators. Pollination structures of flowering plants are relatively less varied compared to vegatative plant structures, for fit between pollinator and pollinated organ and therefore efficient pollination (Armbruster, 2009; Pelabon 2014). Therefore, the behaviors of organisms and the physical interactions of morphological traits with the environment and other organisms impose selection on the scaling relationships of the structures to body size or to other traits (Shapiro and Porter ,1989; Eberhard, 2011).

1.2 Scaling in Bumblebees

Bumblebees (Hymenoptera: Apidae: *Bombus* spp.) are key pollinators of crops and wildflowers that are essential for agriculture and healthy ecosystems (Goulson, 2010). As important pollinators, bumblebee mouthparts have evolved along with flowering plants to allow for a liquid-based diet and pollination. Bumblebees are commonly known to be generalist pollinators that feed on nectar and pollen from a wide range of flowering plants (Heinrich, 1979; Goulson, 2010). However, unlike other eusocial bees, such as honeybees, bumblebees are unique in their high degree of individual body size variation, with individuals from the same hive varying up to 10-fold in mass (Couvillon et al., 2010). This size variation allows differently sized workers from the same species to specialize in foraging on different Bumblebee tongue length, size, and strength all affect flower access and so flower choice (Willmer, 2011). However, this flower choice and division of labor does not only occur within species. Different bumblebee species also vary in size and tongue length, which influences their foraging niche flowers that are suited more to their body size (Goulson, 2010; Peat et al., 2005). This variation suggests that all bumblebee species may not be true generalists, or that different species may be more specialized than others. Variation in body size and mouthpart structure and size differences between species may also play a role in bumblebee flower preference. Indeed, several studies have found that bumblebee species tend to forage on flowers that are appropriate for their mouthparts, specifically their proboscis length (Pyke et al., 1982; Pyke et al., 2012).

Tongue length of bumblebees, both individually and as a species, are critical to their flower choice. In general, bees forage nectar from flowers that have corolla depths that match their tongue lengths (Heinrich 1979). Long-tongued species such as *B. hortorum* and *B. consobrinus* tend to take nectar from flowers that cannot be visited by other foragers in the area due to their shorter tongue length. On the other hand, short-tongued species such as *B.lucorum* and *B.bifarius* tend to visit flowers with short tubular corollas and bowl-shaped flowers. They are also known to nectar rob longer tubed flowers by cutting holes on the side of the flowers with their mandibles instead of using their tongues to reach into the flower corolla to collect nectar (Willmer, 2011).

Although body size, mouthparts, and foraging behavior have been previously studied separately, or in combination with other factors, the relationship between these elements have been given little attention. In addition, studying the bumblebee mouthparts as a whole have been disregarded in favor of studying solely their glossa or proboscis, a specific structure of the bee tongue, since they are the main structures involved in nectar collection (Willmer, 2011). However, I would like to study the whole structure of the mouthparts as they are also potentially involved in nectar collection and may play a role in fitting overall mouthpart shape to flower preference.

1.3 Bumblebee mouthpart anatomy

Bumblebee tongues combine a lapping capillary tip with a suctorial tube and are similarly constructed to other bee tongues while mainly differing in proportions of different sections

(Willmer, 2011; Krenn et al., 2005). Bumblebees use their flabellum, a specialized glossa terminal that has a hairy surface, to initially lap up the nectar. The glossa also forms a channel that leads to the maxillae, where the suction occurs. The galea surrounds the maxillae and elongates the food channel. In bumblebees, the glossa is specialized with hairs that can be erected or laid flat as the rod inside the glossa retracts or extends to absorb nectar. Tongue length varies allometrically in relation to body size both between individual bees and between different species (Willmer, 2011; Borrell and Krenn, 2006).

1.4 Bumblebee body size and behavior in relation to mouthparts

In general, because bumblebees had to adapt to interact and collect nectar from flowering plants, I expect that bumblebee mouthparts may have been under stabilizing selection throughout evolutionary history. Consequently, bumblebee mouthparts may generally follow similar trends to insect genitalia and the lock-and-key hypothesis, with mouthparts having reduced variability and shallow scaling in size and shape relative to body size. In addition, their high variation in body size and specialization of individual bees and species on different flower species may have placed different constraints on different species of bumblebees.

With the overarching hypothesis that variation in body size allows for different bumblebee individuals and species to specialize and forage on different flowering species, a few predictions can be generated. Generalist bumblebees that forage on a variety of flower species may have large variation of mouthpart shapes and close to isometric scaling of their mouthpart trait size to body size to allow for division of labor among individuals foraging on a diverse range of flowers. On the other hand, specialist bumblebees that forage on specific flower species may have lower variation in mouthpart shapes and canalized scaling of mouthpart size to body size, so that all individuals can forage on flowers with similar morphologies. If these predictions hold true, then it would also be possible that nectar robbers that do not interact directly with the flowers' corollas are freed from the constraints to match their mouthparts to the flowers and therefore show even more variation and steeper scaling than generalist species.

1.5 Wings

Another important morphological structure in bumblebees are their wings. Bumblebees have two forewings and two hindwings that they use to fly in a similar fashion to hovering bees but with slow forward flight and not motionless hovering (Joos et al., 1991; Skandalis and Darveau, 2012). Like other body traits, we hypothesize that both forewings and hindwings will scale with body size in Bumblebees. However, since they are not directly related to pollination and therefore may have not been under the stabilizing structure that mouthparts had, our null expectation is that their variance in wing length and shape may only differ according to phylogeny between species (Gérard, 2020). Therefore, these traits were used as the reference trait to compare against the mouthparts. I expected to see more variation in shape and steeper scaling to body size than mouthpart traits due to the nonexistence of the stabilizing selection that are on mouthparts.

1.6 Studying bumblebee mouthpart variation and scaling

To study the mouthpart variation and scaling relationships to body size of different bumblebee species as well as their possible relationship to foraging behaviors, I focused on eight bumblebee species in Maine. These 8 species were the following: *B. vagans, B. impatiens, B. ternarius, B. terricola, B. borealis, B. bimaculatus, B. sandersoni, B. fervidus*. Maine is a great place to conduct this study because of its diverse geography and bumblebee species, with 17 recognized bumblebee species, 10 of which my lab was able to collect and identify through our survey efforts since the summer of 2017. However, because of specimen numbers, only eight different species were used in the study.

With eight species of bumblebees collected around Maine, multivariate morphometric analysis was conducted on their mouthparts using linear measurements across different mouthpart structures. In addition to the mouthparts, the wings were also studied as a reference and contrast morphological structure.

Overall, the objective of this study is to examine the scaling relationships between body size and mouthpart structures, the variation in mouthpart shape, and determine whether there is a relationship between these patterns and foraging specialization of different bumblebee species. Through this, I hoped to gain a better understanding of different patterns in scaling relationships and variation in morphology found in nature as well as how function of a trait and behavior can create evolutionary constraints that lead to such patterns. Here, I report the implications of bumblebees adaptation to forage on flowering plants on their mouthpart morphology. My data suggests that the stabilizing selection on bumblebee mouthparts did constrain their morphology to have low variation in shape and shallow scaling to body size. In addition, different bumblebee species had different mouthpart morphologies and amounts of variation and scaling to body size depending on their foraging specialization. More specialized bumblebees such as *B.ternarius* and *B.terricola* had lower variation in mouthpart shape and shallower scaling of tongue to body size than generalist species such as *B.impatiens*, *B.bimaculatus*, and *B.vagans.*

2. MATERIALS AND METHODS

2.1 Collection of bumblebee specimens

In total, 739 bumblebees of 10 species were collected around Maine starting from the summer of 2017 to 2020. Bumblebees were collected in various places in and islands around Maine. The bumblebees were collected in small plastic vials, imaged with a ruler for scale in a petri dish, identified, and immediately put into freezer boxes (Williams et al., 2014). After collection from the field, they were stored in the -80 ℃ freezer to allow for euthanization and preservation.

In general, males tend to have smaller and fuzzier bodies, with fur under their abdomen and on their mandibles. In addition, their mandibles are visibly smaller and only two pronged compared to the 5 pronged mandibles of the females. The female queen and worker bees were distinguished by their size and time of year. Queens are notably larger than workers and tend to appear more frequently earlier in the year after hibernation in late winter or spring to look for nest sites. After a few days of appearing, the queens stop foraging and there are only workers that collect nectar and pollen from the fields. Then between April and August, depending on the species, the nest starts rearing males and new queens, which can also be seen foraging in the field (Goulson 2010).

2.2 Dissection and imaging of bumblebee mouthparts and wings

The mouthparts and wings were dissected from the body and imaged to be studied. Dissection of the wings was carried out by cutting the joints connecting the wings and the body. The wings were then mounted on glass slides. The mouthpart dissections were conducted after removing the head from the body. Then, with forceps, the structures were folded out from under the head and the connecting tendons and soft tissue were cut. The mouthparts were stored in 100% ethanol at -20 °C.

Both structures were imaged using a VWR VistaVision light microscope connected to a Moticam 5 digital camera and imaged using Motic Images Plus 3.0ML and Motic Images Devices. The wings were oriented with the tegula to the left. The mouthparts had to first be mounted on a slide with double-sided tape to make sure the structure was laid flat and spread out as much as possible.

2.3 Digitization and analysis of mouthparts and body size

To investigate the different mouthpart structure variation and scaling relationships to body size between species, the mouthparts and body size were digitized using ImageJ. The line tool was used to measure the width and lengths of the different mouthpart structures. To try to capture the shape of the mouthparts, I made 15 linear measurements across different structures (Fig. 1D). No landmarks for geometric morphometrics could be placed because the mounting and imaging of the mouthpart structures were too inconsistent between specimens due to their flexible and hinged structures.

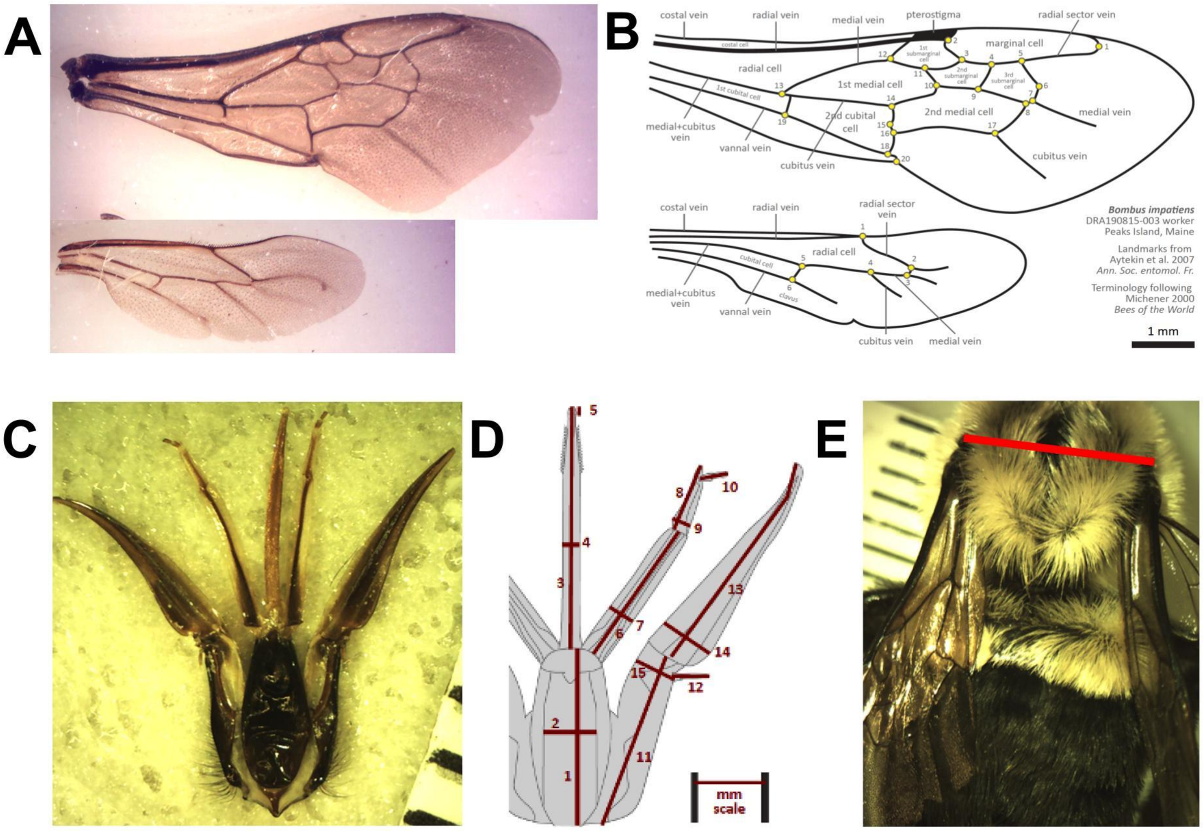


Figure 1. *Bombus* Morphological structures and methods of measurement. (A) *Bombus* forewing and hindwing. (B) Landmarks on the Bombus forewing (20) and hindwing (6) for conducting landmark based geometric morphometrics. (C) *Bombus* fullmouthpart structure. (D) 15 linear measurements on the mouthpart to conduct multivariate morphometric analysis. (E) Measurement of the *Bombus* intertegular span (its).

The line tool was also used to measure the body size of the specimen. One method was to measure in segments, the head, the thorax, and the abdomen and combine the measurements for a full body size. I also measured the intertegular span (ITS), the dorsal span between the two wing hinges (Fig. 1E). All measurements were exported in pixel numbers and converted to metric measures of length based on the ruler scale picture taken with the specimen. This method allowed for the comparison of all lengths across different bumblebee species as well as castes. For final analysis of the data, ITS measurements were used instead of the full body size measurements for the following reasons. One, bumblebee bodies dry out and shrink as time passes, causing full body size measurements to be inaccurate depending on the time the image was taken and measured. Two, using ITS measurements as a stand in for body size is a common practice among bumblebee scientists (Mola et al., 2020). Three, both analysis using full body size and ITS measurements resulted in similar patterns, but showed clearer trends when ITS measurements were used.

Scaling of tongue length to body size and the multivariate mouthpart shape variation between individuals and species was visualized by principal component analysis (PCA). All statistical analyses were based on permutation multivariate anova (permanova) using the R package RRPP (Collyer and Adams, 2021). The common package used for permanova, vegan, was not used because it uses diversity-based distance matrices appropriate for ecology datasets. RRPP is a more appropriate package to use for studying morphometrics because it employs residual randomization and supports multi-factor models. Differences in species were investigated using post hoc pairwise comparisons through the function rrpp::pairwise. During the analysis, the Z statistic was the measure of effect size, which is calculated as the standard deviations of the F value that is log-transformed first to create normally distributed data (Adams et al., 2021). In addition, all analyses were performed with only worker data, as there was very unbalanced sampling between the different castes of different species and can be a confounding factor.

To gain a better understanding of the relationship between mouthpart shape and foraging behavior of bumblebees, the relationship between mouthpart shape and a quantitative measure of foraging behavior was tested using ANOVA. The foraging behaviors of different bumblebee species were quantified by calculating different metrics using the forage plant data found in

Wood et al. 2019. The different metrics are the following: dietary breadth, Simpson's diversity index, PC1, and PC2. Dietary breadth is a metric calculated by Wood and colleagues that represents the number of pollen types each bumblebee species is expected to collect. They identified pollen from 811 museum bumblebee specimens of 12 different species in Michigan from 1912 to 2019. The other metrics were derived from the raw forage plant data of different species presented by Wood and colleges in the form of pie charts. The pie charts reported the relative contribution of different plant species to the pollen load of each bumblebee species. The number of specimens for each species that collected pollen from each plant taxon was determined based on the total sample size for the species and each fractional area. This resulted in a species interaction matrix, which was scaled and centered before conducting principal component analysis. The first principle component focused on one plant taxon Fabaceae, and so I used the second component PC2, which captured a greater diversity of plant taxa. The Simpson's diversity index is a measure of diversity that takes into account both the number of species and the number of individuals in each species. A high Simpson's diversity index means that there is both higher species richness, number of species, and evenness.

2.4 Digitization and Analysis of wings

To investigate the variation of wing shapes and sizes between species, the dissected wings were mounted onto glass slides, imaged, and digitized using ImageJ. To capture the variation in both size and shape of the different bumblebee species wings, landmark-based geometric morphometric (GMM) analysis was conducted. Using ImageJ, 20 points were placed on the forewing wing vein intersections and 6 points were placed on the hindwing vein intersections. All landmarks were chosen based on homology among individuals (Fig. 1B). Then, the coordinates of these landmarks were exported into an Excel spreadsheet and the cartesian coordinates were converted into TPS format using the function create.tps in the R package borealis (https://github.com/aphanotus/borealis).

For analyses, the point coordinates were imported into R and processed using the packages “geomorph” (Adams et al., 2021) and “borealis”. All landmarks were considered to be fixed landmarks. Specimens were aligned using Generalized Procrustes Analysis (GPA). Outlier analysis was conducted and outliers removed if they were clearly due to errors in digitization, such as specimen with landmarks placed out of order. Shape differences between species were visualized by PCA. To compare shape and size variation between the wings of bumblebee species, modeling was carried out using permutation-based Procrustes ANOVA and the function ‘procD.lm’. Post hoc pairwise comparisons were also conducted using the function rrpp::pairwise. In addition, phylogenetic PCA as well as phylogenetic ANOVA was conducted to study whether similarity between species were due to a shared evolutionary history or convergence. To do so, a phylogeny based on the unpartitioned sequence of five genes reported from Cameron et al. 2007 was produced using RAxML version 8.2.11 on a multiprocessor computing cluster. Mutation rates were estimated from a GTR model with a gamma distribution and portion of invariant sites. Disparity comparisons among different species were conducted using the function morphol.disparity in the R package geomorph (Adams et al., 2021).

Analyses were performed on both forewings and hindwings. However, as the trends are more pronounced in forewings, only the forewing results are reported below. In addition, although analysis was performed using all castes, because there was unbalanced sampling between the castes of all the species, only analysis done using workers are reported below.

2.5 Relative Scaling

To study the relative scaling of the focal trait, mouthparts, the wings were used as the reference traits. The variation in both tongue length and tongue length scaling to body size was compared to variation in forewing and hindwing length and wing scaling to body size.

3. RESULTS AND DISCUSSION

3.1 Forage Plant Diversity and Specialization

To find the relationship between forage plant diversity and specialization of different bumblebee species, different forage plant diversity metrics calculated from forage plant data from Wood et al. 2019 were used. While the relationship between mouthpart morphology and forage plant diversity will be explored in future sections, I was able to discern a few things. Common species that can be seen in locations all throughout Maine and known to be generalist species, such as *B. impatiens*, *B. bimaculatus*, and *B. vagans* all fell highly on all diversity indexes (Fig. 2A). High values on all indexes denote higher forage plant diversity or count and therefore a generalist strategy. On the other hand, other more uncommon species such as *B. ternairus*, *B. terricola*, and *B. borealis* fell inconsistently, but usually lowly on all diversity indexes. These species may be specialists that place inconsistently on different metrics due to the fact that there are many different ways to define and quantify specialization. Bumblebees can specialize in different ways. Different species of bees may forage on the same number of flowering plant species but with different evenness. Alternatively, different species may forage on the same number of flowering plant species but species with different variations in morphology. Therefore, while *B.ternarius* and *B.terricola* may forage on only a few different flower species and have low values in both dietary breadth and PC2, they may have high evenness among these forage plants and so have a high Simpson's diversity index (Fig. 2A).

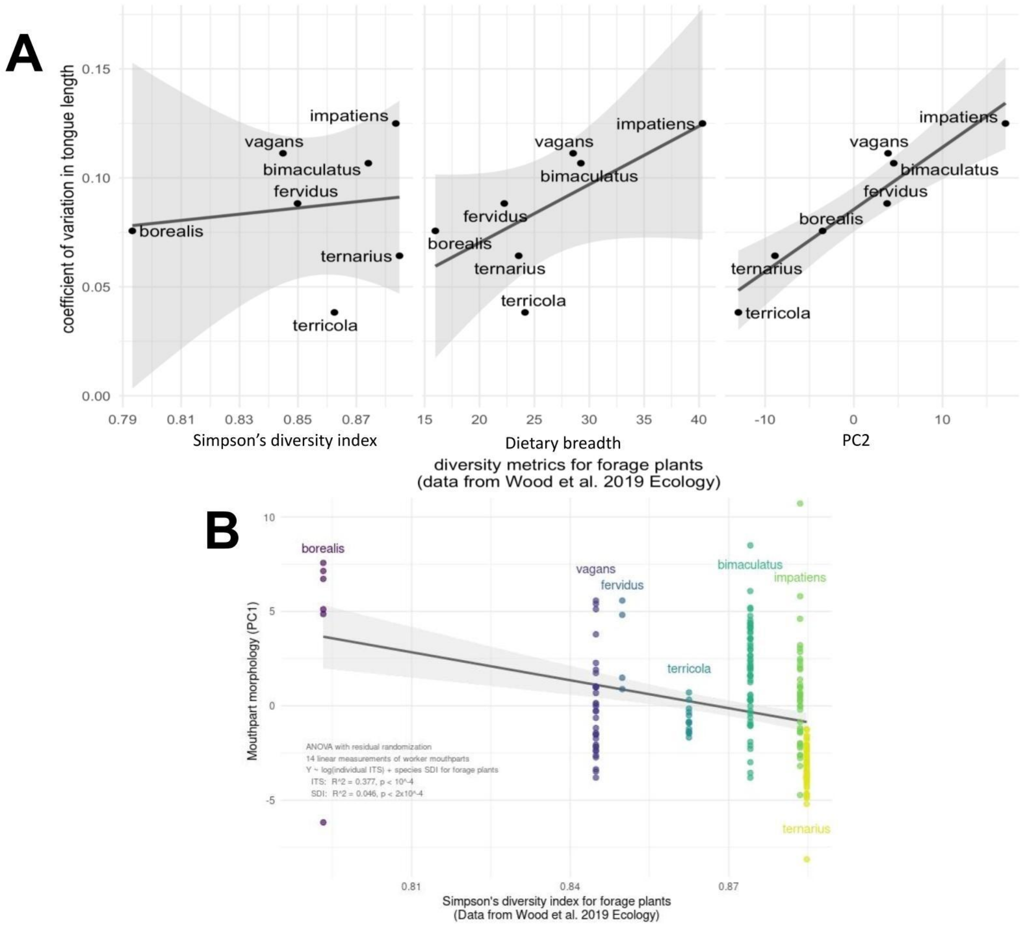


Figure 2. Correlation between foraging specialization and mouthpart shape and variation. (A) There is significant correlation between the coefficient of variation in tongue length to different diversity metrics for forage plants all calculated from forage plant data of different species from Wood et al. 2019. PC2 showed the strongest correlation, but all other metrics such as Simpson's diversity index and dietary breadth also had significant correlation to forage plant diversity. Generalist species tend to fall on the right of the graph while the specialist species tend to fall on the left side of the graph. (B) There is significant correlation between PC1 of mouthpart shape and Simpson's diversity index for forage plants.

3.2 Wings

Bumblebee wing shape was studied as a reference to compare mouthpart shapes to. For bumblebee wings, I expected more variation in shape and steeper scaling of wing length to body size.

3.2.1 Wing length

All parts of an organism scales in one way or another to body size. When forewing length and intertegular span (ITS) of workers of different species was plotted, all species showed proportional scaling to body size with similar slopes (Fig. 3). However, when differences in slopes based on overlap of CI were analyzed, it showed that *B.bimaculatus, B.ternarius, B.terricola,* and *vagans* had the same slope while *B.impatiens* had a different slope and the rest of the species, *B.Borealis* and *B.fervidus* had slopes overlapping all species. Overall, most of the species showed similar scaling of wing length to body size, which may be due to the fact that their wing length is related to the aerodynamics of the wings and wingstroke frequency. Therefore, wings may be under stabilizing selection for length as all species need similar wing sizes on individuals with similar body sizes to allow for flight.

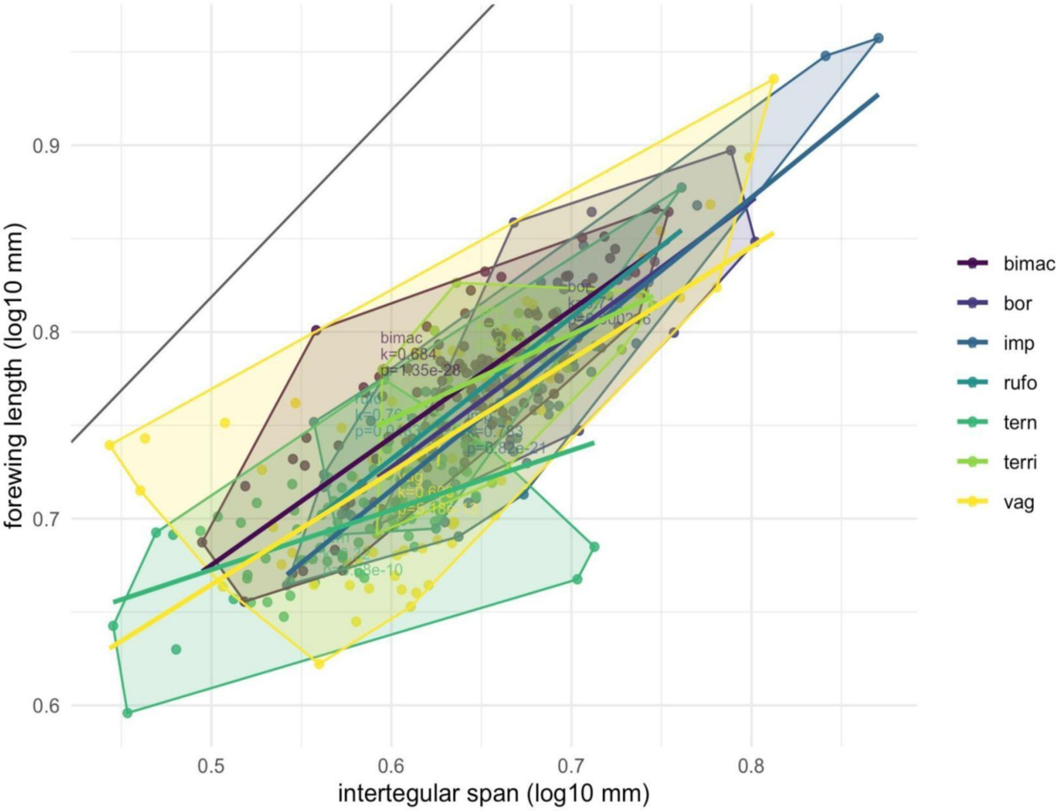


Figure 3. Morphospace plot of forewing length to intertegular span of different bumblebee species. Most species show similar slopes with the exception of *B. rufocinctus* and *B. ternarius* that have shallower slopes.

3.2.2 Wing Shape

3.2.2.1 Wings Principal Component Analysis (PCA)

Therefore, PCA of workers were also performed and the resulting first two PC axes were PC1 accounting for 26.4% of the variance and PC2 accounting for 8.3% of the variance (Fig. 4). The other resulting PC axes, PC3 and PC4, accounted for 9.24% and 7.24% of the variance, respectively. The morphospace plot shows the different distribution of species wing shapes as well as example shapes from each axis. Through the plot, it can be seen that PC1 separates wings that are broad from those that are narrow. PC2 separates wings that have a distal anterior skew to those that have a proximal anterior skew. The plot also shows that more common species such as *B.vagans* and *B. bimaculates* have greater shape disparity, or more variance in shape, than species such as *B. borealis* and *B. terricola*. In addition, *B. borealis* and *B.fervidus* have notably lower values of PC1, PC2, and PC3, showing separation from the different species.

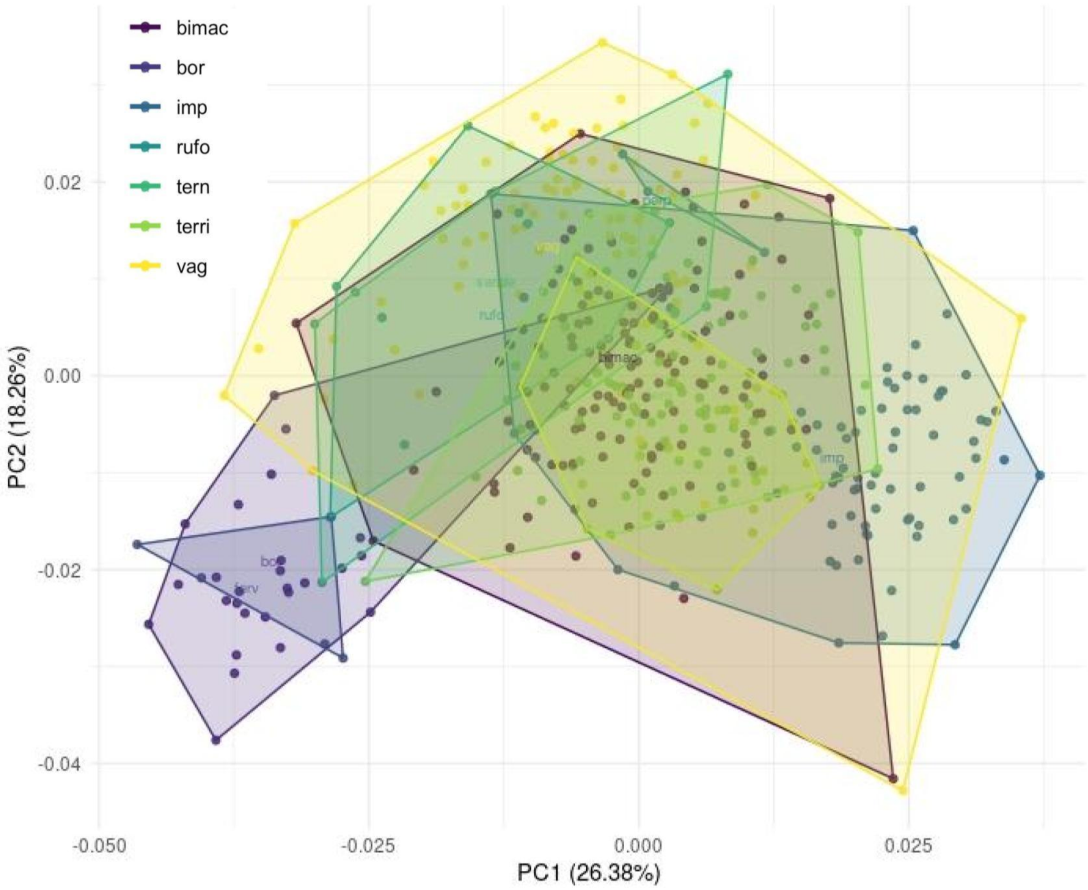


Figure 4. Overall analysis of *Bombus* forewing shape. Most species occupy the same regions in the shape space. Common species such as *B. vagans* and *B. bimaculatus* occupy larger areas of the morphospace than other species while *B. borealis* and *B.fervidus* have notably lower PC1 and PC2 values than the other species.

3.2.2.2 Morphological Disparity Comparisons

Different species took up different areas of the shape space morphospace, indicating that the extent of variation in wing shape between organisms (disparity) may be different among different species. To test this, a disparity test was conducted, which showed that *B.impatiens* (0.001)*, B. vagans* (0.001)*,* and *B. borealis* (0.002) had higher disparity variances among workers than *B. ternarius* (0.00057), *B.bimaculatus* (0.00064)*,* and *B.terricola* (0.0007). However, in general, all the species had low disparity variances.

3.2.3 Modeling

3.2.3.1 General Modeling for Wing Shape

For the linear modeling on bumblebee forewing shape, centroid size (Csize), ITS, species, and castes were tested as predictors. Only species with more than 10 specimens in all castes were used for analysis. In a model including all predictors (coords ~ log(Csize) + species + caste), species has the largest effect size on forewing shape based on Z-scores (permutation-based multivariate ANOVA, $F(6,571) = 44.57$, $Z = 15.13$, $p = 0.001$) . In addition, centroid size ($F(1,571) = 75.23$, $Z = 7.86$, $p = 0.001$) is also a strong influence on shape , while caste ($F(2,571) = 5.26$, $Z = 4.31$, $p = 0.01$) has the weakest effect, although it is highly significant. Post hoc pairwise comparisons for species showed that forewing shapes of different species are all different, except for *B. rufocinctus* and *B.vagans.* This may be due to the low sample size of *B.rufocinctus* due to the inability to easily distinguish the species.

3.2.3.2 Common Wing Allometry Among Workers

To compare the allometry of wing shapes to ITS of different species workers, the ITS was plotted against PC1 for worker forewing shape (Fig. 5). While most species showed similar scaling, *B.terricola* and *B.rufocinctus* had flat slopes with confidence intervals that spanned zero. When different allometric models using ITS measurements were compared (coords ~ log(ITS) + species; coords ~ log(ITS) * species), ITS and species interaction term is a significant factor influencing wing shape (F(9, 404) = 1.71, Z = 3.08, p = 0.002). In other words, different species have unique allometries. Pairwise comparisons of species-specific allometric slopes showed that all species, *B.bimaculatus*, *B.borealis*, *B.impatiens, B.terricola,* and *B.vagans,* had unique allometry except *B.rufocinctus,* and *B.ternarius*.

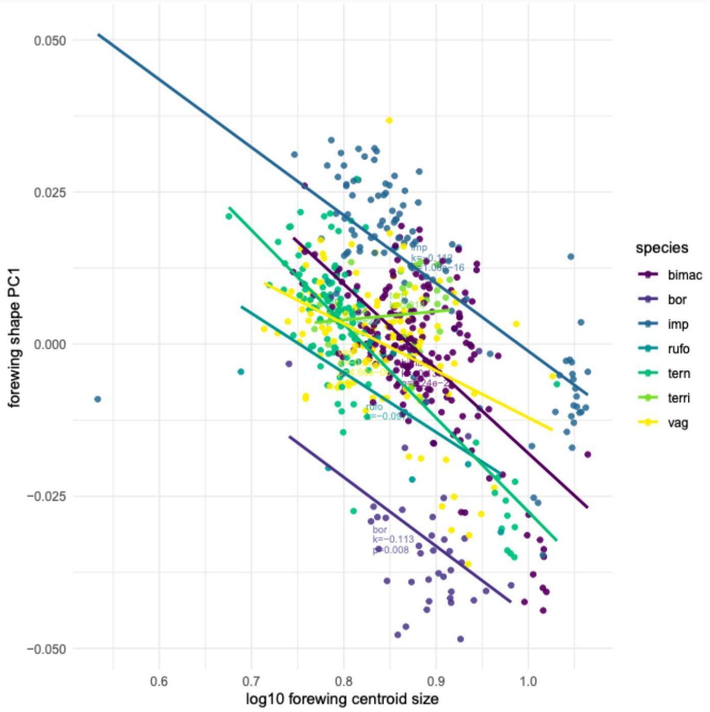


Figure 5. Allometry of forewing shape to intertegular span of different *Bombus* species. Most species show similar scaling. However, *B.terricola* and *B.rufocinctus* have shallower slopes.

3.2.4 Phylogenetically aligned PCA and Phylogenetic ANOVA

Most of the species shared similar and overlapping areas in the shape space morphospace (Fig 4). Therefore, to test whether the similarity is due to a shared evolutionary history or to convergence, the ordination of shape data along phylogenetic relationships was looked at. Using the phylogenetic tree from Cameron et al. (2007) and mean wing shapes of each species, the shape space was plotted using the first two PC axes, PC1 (60.45%) and PC2 (23.07%) (Fig. 6). Through this plot, it can be seen that the aligned PCA is very similar to the regular PCA. In addition, the two long-faced bees, *B.borealis* and *B.fervidus* occupy distinct parts of the morphospace apart from the rest of the species that corresponds to their divergence from the other species. In this analysis, when phylogenetic ANOVA was conducted, phylogeny did have an influence on wing shape. When the phylogenetic signal was removed, the influence of neither centroid size or ITS was a significant influence on shape (Csize: F(1,8) = 1.37, Z = 0.68, p = 0.25; ITS: F(1,8) = 1.53, Z = 0.86, p = 0.20).

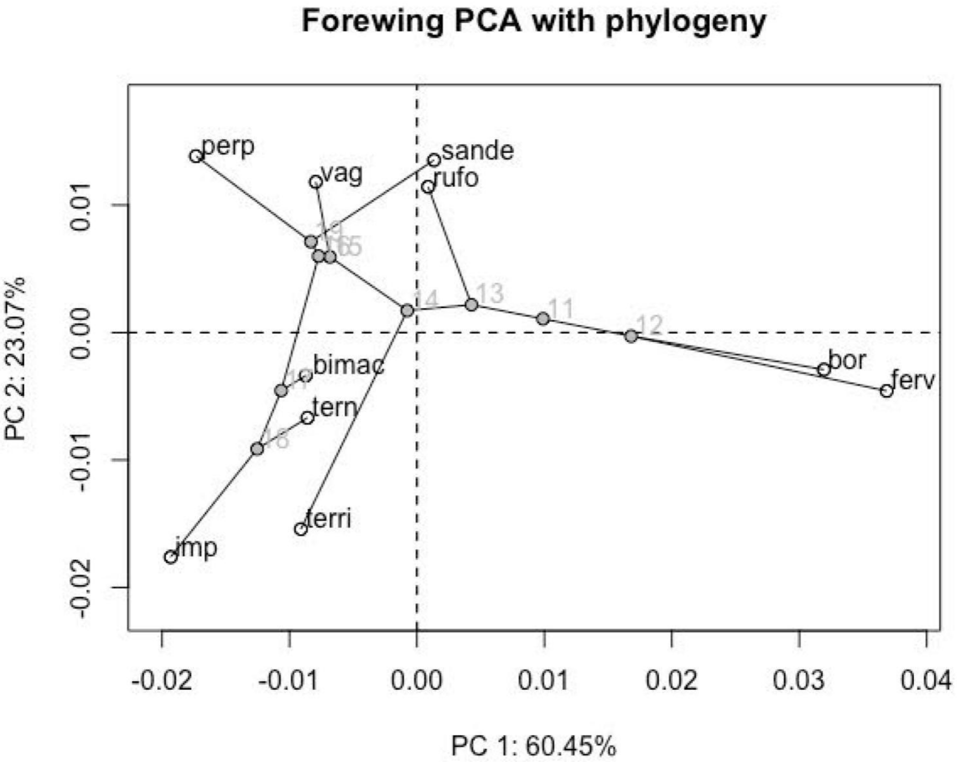


Figure 6. Forewing PCA with phylogeny shows that *B.borealis* and *B.fervidus* both occupy different regions of the morphospace than the other species, which corresponds to their divergence.

3.3 Mouthparts

3.3.1 Tongue Length

Tongue scaling to body size using ITS measurements shows that different species workers have different scaling coefficients. On the extremes, *B.borealis* has both higher variation and steeper scaling while *B.ternarius* and *B.terricola* have lower variation and smaller scaling coefficients than other species such as *B.vagans*, *B.impatiens* , and *B.bimaculatus* (Fig. 7). It was hypothesized, that because *B.ternarius* and *B.terricola* have lower scaling of tongue length to body size, and therefore consistent tongue sizes among all individuals regardless of body size, that these species might be specialists that have consistent mouthpart morphology in all individuals so that every individual may forage on similar kinds of flowers.

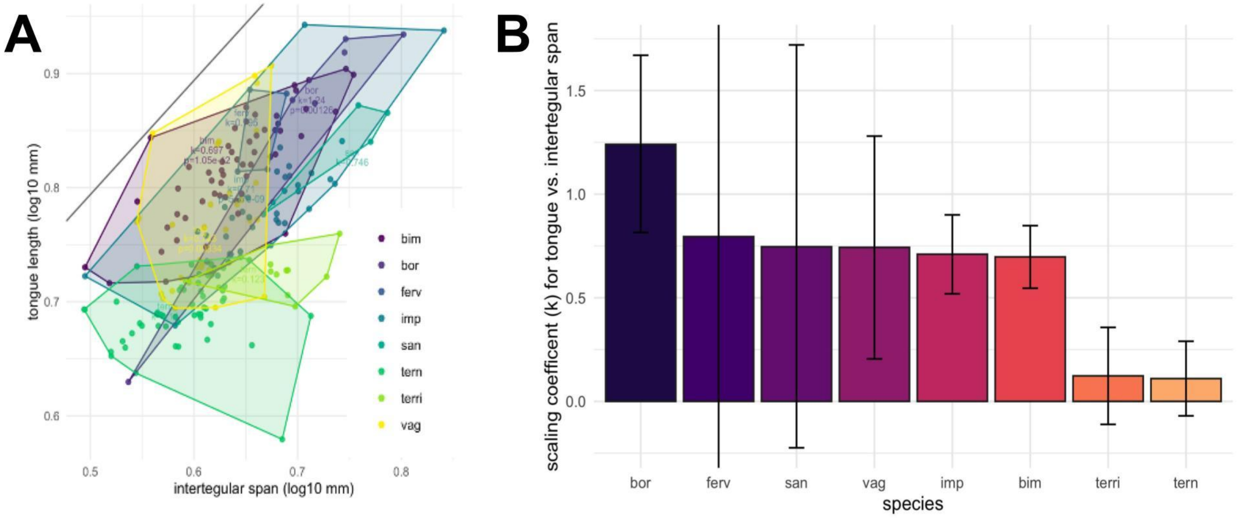
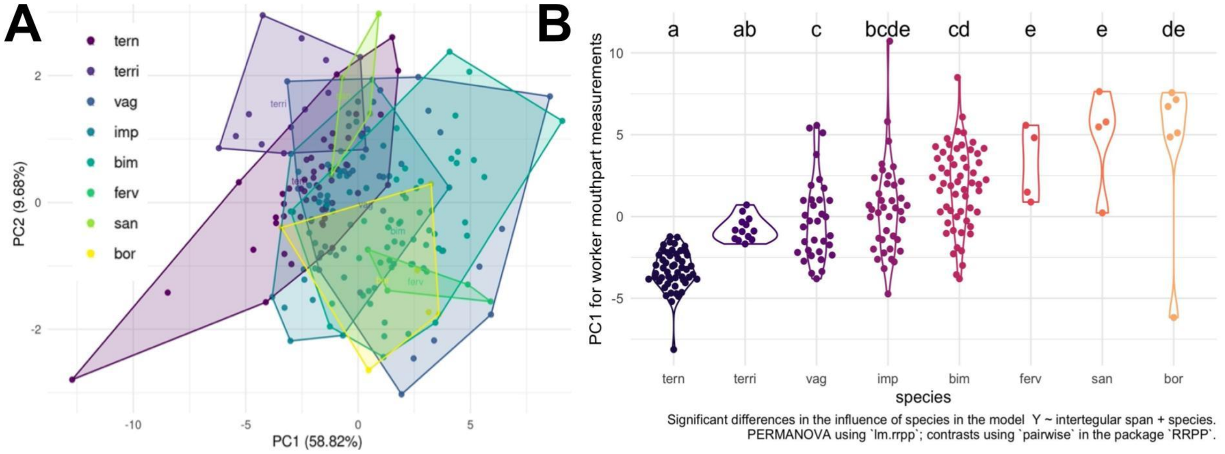


Figure 7. Analysis of *Bombus* tongue scaling to body size. (A) Bombus tongue scaling to log of intertegular span plotted in morphospace. From the plot, it can be seen that different species have different scaling slopes. (B) The scaling coefficient of different *Bombus* species. *B.ternarius* and *B.terricola* have shallower slopes than other species such as *B.vagans*, *B.impatiens*, and *B.terricola*.

3.3.2 Mouthpart Shape

3.3.2.1 Principal Component Analysis

Linear multivariate mouthpart measurements were used to represent and study "shape" because no consistent landmarks were able to be placed, and therefore GMM could not be conducted, due to the flexibility of the mouthparts. Morphospace plotting PC1 (73.39%) and PC2 (6.66%) shows that most species overlap and occupy similar regions of the morphospace (Fig. 8A). Disparity comparisons were unable to be conducted between the different species because a method to conduct a multivariate test of group variance implemented in R could not be found.



Significant differences in the influence of species in the model Y ~ intertegular span + species.
PERMANOVA using `lm.rrpp`; contrasts using `pairwise` in the package `RRPP`.

Figure 8. Analysis of overall *Bombus* mouthpart shape. (A) Morphospace of *Bombus* mouthparts distinguished bys pieces. Generally, all species overlap in space with the exception of *B.ternarius* and *B.terricola* that are slightly off to the left of PC1. (B) PC1 differences in *Bombus* species with pairwise difference significance denoted as letters above the violin plot. As seen in the morphospace, *B.ternarius* and *B.terricola* showed similar PC1 separate from the rest of the species.

3.3.3 Modeling

3.3.3.1 Modeling for Mouthpart Shape

All statistical analysis and modeling was conducted using the package RRPP (Collyer and Adams, 2021) and workers from species with more than 10 specimens. For generalized linear modeling of mouthpart shape (PC1), ITS, and species were tested as predictors (mm ~ log(ITS) + species. The model showed that species has a larger effect on overall mouthpart shape based on Z-scores (Permutation based multivariate ANOVA: $F(7,198) = 31.08$, $Z = 10.66$, $p < 10^{-4}$) than ITS ($F(1,198) = 267.3$, $Z = 5.46$, $p < 10^{-4}$), although both were significant. In addition, pairwise comparisons showed that there are indeed species differences in shape (Fig. 8B). *B.ternarius* and *B.terricola* showed similar PC1 separate from the rest of the species while

B.impatiens overlapped with all species except *B.ternarius*. In addition, *B.vagans, B.impatiens, B.bimaculatus* showed overlap that was distinct from other species.

3.3.3.2 Mouthpart shape allometries

To determine whether different species have different mouthpart "shape" allometries a model with species and body size as an interaction term was tested (mm ~ log(ITS) * species). Using this model, it was concluded that different species indeed do have different worker mouthpart "shape" allometries ($F(7,191) = 6.76$, $Z = 3.92$, $p < 1^{-4}$). In addition, a post hoc examination of the interaction showed that there are interesting differences among the allometries. *B.terricola* and *B.ternarius* have similar allometries to each other ($Z= -1.38$, $p = 0.91$) and are different from other species. In addition, all other species, such as *B.vagans*, have similar allometries to *B.impatiens* and *B.borealis*. However, *B.impatiens* and *B.borealis* have significantly different allometries from each other ($Z = 1.87$, $p = 0.025$).

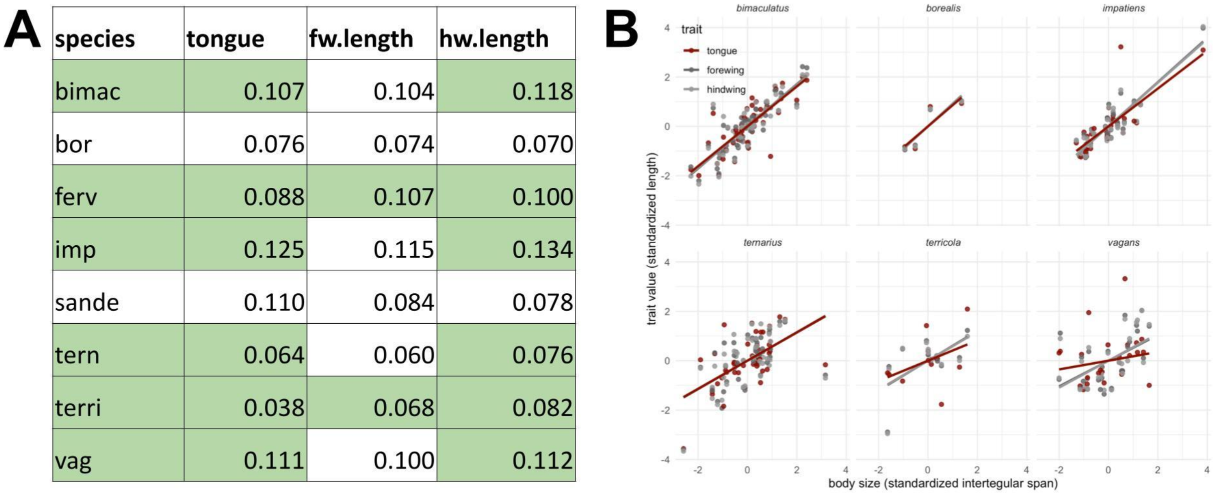
3.3.4 Phylogenetic Approach

Like the wing analysis, both phylogenetic PCA and phylogenetic ANOVA were attempted on the mouthparts. However, I was unable to do this due to limitations in the functions.

3.4 Relationship of Wings and Mouthparts to Forage Specialization

3.4.1 Relative Scaling

To actually compare the mouthpart allometry to the allometry of the reference trait, the wings, I combined the dataset and analysis of each trait. In general there was lower variance in mouthpart shape than wings in some bumblebee species, as well as shallower scaling to body size of tongue length compared to the scaling of both forewing and hindwing length to body size (Fig. 9). However, none of the results were significant and therefore does not support the hypothesis that since there is stabilizing selection on the mouthparts due to the need to adapt to foraging on flowering plants, mouthparts must have lower variance in shape and shallower scaling of traits to body size. This nonsignificance in results may be due to a few different reasons. Perhaps the hypothesis is truly wrong and there is no actual stabilizing selection on the mouthparts, and so no difference between the mouthpart traits and the wings can be seen. Or, perhaps, the hypothesis is true and there is stabilizing selection on the mouthparts, but because wings need to allow for flight of bumblebees, there is also different stabilizing selection that acts on the wings. Therefore, wings may not be a good reference trait to use to compare other focal traits to use. Instead, other traits such as the more traditionally used bumblebee antenna or leg should be used to compare against both the mouthpart structures and the wings.



species	tongue	fw.length	hw.length
bimac	0.107	0.104	0.118
bor	0.076	0.074	0.070
ferv	0.088	0.107	0.100
imp	0.125	0.115	0.134
sande	0.110	0.084	0.078
tern	0.064	0.060	0.076
terri	0.038	0.068	0.082
vag	0.111	0.100	0.112

Figure 9. Comparisons between mouthpart shape variation and trait scaling to wing shape variation and scaling to body size. (A) Variation in mouthpart shape was lower for mouthparts than for forewings or hindwings in some *Bombus* species. (B) Variation in tongue length scaling to body size show trends of being lower than for variation in forewing length scaling to body size for all *Bombus* species. However, none of these results were significant.

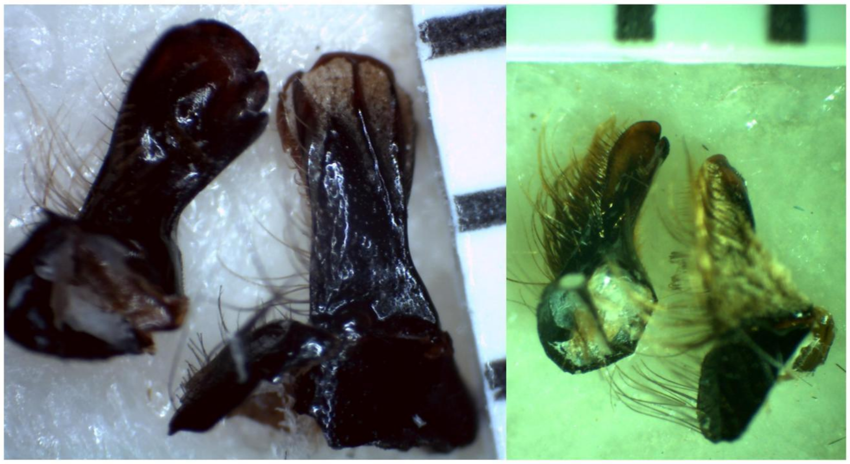
3.4.2 Mouthpart shape scaling to forage diversity metrics

After finding patterns in mouthpart shapes of different species, to explore the hypothesis that different foraging behaviors of different bumblebee species would have placed different constraints on mouthpart morphology over evolutionary time, I explored correlations of mouthpart shape and variation to forage diversity (Fig. 2). When modeling using mouthpart shape data, forage diversity metric, species, and ITS as factors, it was found that there was correlation between shape and foraging diversity metric in all cases, but PC2 from Wood et al. 2019's forage plant data is the best predictor of mouthpart shape ($F(1,201) = 48.44$, $Z= 3.77$, $p < 1^{-4}$) after species ($F(6,196) = 36.39$, $Z = 10.24$, $p < 1^{-4}$) and ITS ($F(1,201) = 121.76$, $Z = 4.82$, $p < 1^{-4}$) (Fig. 2A). However, among the diversity metrics, Simpson's diversity index was the best predictor ($F(1,201) = 22.7$, $Z = 3.08$, $p < 1^{-4}$). In general, the correlation between shape and foraging diversity metric gives support to the hypothesis that different species may have different mouthpart morphology and scaling to body size based on their forage behavior.

4. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, I was able to find that there is significant correlation between morphological variance in mouthparts and foraging specialization in bumblebees! The trends seem to show that specialist bumblebees such as *B.ternarius* and *B.terricola* that score lower on different diversity metrics than generalist species such as *B.bimaculatus*, *B.impatiens,* and *B.vagans* have shallower scaling of tongue lengths to body size and lower variation in mouthpart shape. In addition, although it could not be proven statistically significantly, there seems to be a trend that mouthparts show shallower scaling to body size as well as lower variation in shape than the wings, which would be what is expected of a structure under stabilizing selection. Therefore, the results indicate that bumblebee mouthparts that adapted to collecting nectar from flowering plants may have been under stabilizing selection like insect genitalia to have shallower scaling and less variation.

In the future, more work could be done to increase specimen size and conduct other measurements of different structures. First, other structures such as the antenna or legs could be measured to use as reference traits to compare both mouthparts and wings to. In addition to these structures not having stabilizing selection similar to those mouthparts and wings are under, it was proposed by O'Brien et al. 2018 that multiple reference traits should be used so that the 'typical' (nonsignal) trait scaling relationship to body size can be captured more accurately and that this exact problem of choosing an inappropriate reference structure will not occur. Second, the specimen size should be increased to have a more even distribution of specimen count between the different castes, workers, queens, and males, so that caste differences in mouthparts can be studied. It is already known that males, unlike female workers and queens, do not contribute to collecting nectar and pollen for the hive, or hive building (Pat Willmer, 2011). In addition, their mandibles compared to the females show stark visible differences (Fig. 10). Males, perhaps because they do not contribute to hive building and therefore do not utilize their mandibles as rigorously as females, have smaller two pronged mandibles with fur. Therefore, their mouthparts may also show different morphology. Third, I would like to study the mouthparts of nectar robbers, which are bumblebees that do not collect nectar in the usual way, but use their mandibles to cut through the flowers and "steal" the nectar. I expect these bumblebees and species that participate in nectar robbing to be freed from the stabilizing selection imposed by need to interact with flowers so that they show increased variation in shape and steeper scaling of their mouthpart shapes to body size. I also would like to see whether their mandibles, that are used even more than normal workers, would show any differences.



B. terricola W

B. terricola W

Figure 10. Mandible size and shape comparisons between workers (left) and males (right). Worker mandibles are larger than the hairy male mandibles.

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APPENDIX

R-scripts and Raw Data

All the raw data collected and analyzed for this project are available to view and download at Colby College's "digital commons" in a folder titled "LeeY_Honors_2021_Supplementary". All R-scripts used for analysis are also available to be viewed and imported into R.