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Pollen composition significantly impacts the development and survival of the native small carpenter bee, *Ceratina calcarata*

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Abstract. 1. As native bee populations decrease, there is a need to better understand their nutritional requirements to sustain healthy pollinator populations. A common native bee to eastern North America is the small carpenter bee, *Ceratina calcarata*. Previous studies have shown that the primary pollen sources for *C. calcarata* consist of clover and rose.

2. The aim of this study is to compare the effects of diet composition on body size, development and survival. Artificial pollen diets were created using five different ratios of commercially available clover and rose pollen.

3. Diets containing higher ratios of clover pollen produced larger individuals with increased survival rates and faster development times. To examine this further, the macronutrient profiles of clover and rose pollen were characterised comparing: protein, sugar, fatty acid, and amino acid content. Results indicated that rose pollen contained significantly higher protein and sugar content, while clover pollen had higher concentrations of essential amino acids. These are crucial to bee health and development, which helps to explain the increased survivorship observed on high clover diet treatments.

4. Taken together, these results show that clover pollen provides a higher quality diet for larval development and survival of the native small carpenter bee. This research indicates that diet has a significant effect on the health of the native pollinator community and more research is needed to further explore the balance between pollen quality and availability, including essential macronutrients and the availability of these floral sources for wild bees.

Key words. Body size, carbohydrates, essential amino acids, fatty acids, small carpenter bees, wild bee nutrition.

Introduction

The nutritional ecology of animals includes optimal proportions of carbohydrates, protein, fatty acids, and essential amino acids. Previous research has shown that insect herbivores have behavioural and physiological adaptations that help them to regulate their intake (Behmer, 2009) and if these targets are not met, it leads to mortality (Pirk *et al.*, 2010; Paoli *et al.*, 2014). Bees rely heavily on nutrition from floral resources, especially pollen and nectar (Michener, 2007). When floral resources are limited or unavailable, bees experience nutritional stress, which can result in population loss and decrease in pollinator efficiency (Biesmeijer *et al.*, 2006; Carvell *et al.*, 2006; Naug, 2009; Goulson *et al.*, 2015).

Pollen requirements vary significantly among bee species and even vary throughout their life cycle. For example, the nutrition of honey bee larvae is significantly different from the nutritional requirements of adult bees. The general nutritional requirements of honey bees have been reviewed extensively by Haydak (1970) and updated by Brodschneider and Crailsheim (2010) highlighting the different requirements of water, protein, and carbohydrates among colony members. Much less is known about

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the general nutritional requirements of wild bees (Woodard & Jha, 2017). There is evidence that some bee species, including honey bees and bumble bees, show foraging preferences based on the nutritional requirements of the colony compared to the nutritional quality of the pollen (Rasheed & Harder, 1997; Kitaoka & Nieh, 2008; Altaye *et al.*, 2010; Kriesell *et al.*, 2016; Vaudo *et al.*, 2018; Ruedenauer *et al.*, 2020). However, other bee species, like *Lasioglossum zephyrum*, are unable to detect specific macronutrient content of plants during foraging (Roulston & Cane, 2002).

The small carpenter bee, *Ceratina calcarata* Robertson (Hymenoptera: Apidae) is a common native bee across eastern North America (Rehan & Sheffield, 2011; Shell & Rehan, 2016). Previous research has demonstrated that *C. calcarata* is one of the most abundant bee species in recently restored ecosystems (Fiedler *et al.*, 2011) and serve as an indicator species for healthy open pine forests with heavy shrub cover (Hanula *et al.*, 2015). *Ceratina* species are generalist foragers that have been shown to be one of the major pollinator species in natural ecosystems, including northern New England (Tucker & Rehan, 2016) and a major pollinator of native plants, like camphorweed (*Heterotheca subaxillaris;* Olsen, 1996), teak (*Tectona grandis* L.f.; Tangmitcharoen *et al.*, 2009) in New England, and geranium (*Geranium thunbergii*) in Japan (Kandori, 2002). In addition to the small carpenter bee's role in native ecosystems, they play a key role in the pollination of many agricultural crops from vegetables and fruit to biofuel production (Tuell *et al.*, 2009; Gardiner *et al.*, 2010; Kennedy *et al.*, 2013; Russo *et al.*, 2013; Tucker & Rehan, 2018).

Previous research has shown that two primary sources of pollen for *C. calcarata* are clover (Fabaceae: *Trifolium* spp.) and rose (Rosaceae: *Rosa* spp.) (Lawson *et al.*, 2016), likely because clover and rose are common resources throughout the foraging season (Tucker & Rehan, 2016). The goals of this study are: first, to compare how types of pollen provided to offspring impact adult body size, development rate, and survival; and second, to compare the macronutrient profiles of pollen from two common plants, rose and clover. Specifically, we compared sugar, protein, fatty acid, and amino acid composition to characterise macronutrients essential for healthy development and survival. By comparing the physiological effects of pollen composition on the development of a native bee species, we can begin to understand the nutritional needs of some wild bee species.

Materials and methods

Collections and nest measurements

Ceratina calcarata nests were collected from staghorn sumac (*Rhus typhina*) branches along roadsides around Durham, New Hampshire, USA (43.1339° N, 70.9264° W) between 5 June and 6 July 2017. Nest collection occurred daily and all collections occurred prior to 8 am to ensure the presence of all nest occupants. After collection, all nests were stored at 4 °C while processed.

Nests were opened longitudinally exposing the contents from end to end (Fig. 1). Brood developmental stage and nest ID were recorded (as in Rehan & Richards, 2010a). Only eggs and first larval stage individuals were used for experiments and one offspring from each nest was randomly assigned to each of the treatments. Eggs were transplanted from the nest randomly onto one of the artificial pollen diets as described below on 96-well PCR plates and stored in a Percival Intellus control system incubator at 25 °C with 50% humidity.

Fig. 1. Inside a *Ceratina calcarata* that has been opened laterally. The nest contains 12 individual brood cells with larvae at different stages of the development, including some feeding on pollen balls. [Colour figure can be viewed at wileyonlinelibrary.com].

Brood was checked every 2 days and the stage of development was recorded. Development was initially tracked by comparing the larval length to the size of the remaining pollen. Following larval development, pupal development was determined using body and eye pigmentation (Rehan & Richards, 2010a). Once adulthood was reached, bees were stored at −20 °C for analysis of sex and head width. Using microscopy with an ocular reticle, head width was measured from eye to eye across the widest part of the head, which has been shown to be an accurate representation of body size (Rehan & Richards, 2010b). The sex of the bee was determined by counting the number of dorsal abdominal plates; females have six and males have seven terga (Rehan & Richards, 2010b).

Artificial pollen diets

Single source white clover (Fabaceae: *Trifolium repens*) pollen pellets collected by *Apis mellifera* purchased from Ames Farm (Watertown, Minnesota, USA; www.amesfarm.com) and single-source rose (Rosaceae: *Rosa* spp.) pollen collected by *Apis mellifera* purchased from the China Health Club (Chongqing, China; www.teaherbalshop.com) were used to create artificial diets to raise *C. calcarata* eggs. Pollen identity and purity were based on vendor claims. Pollen from clover and rose was ground into a powder using a mortar and pestle. Five artificial pollen diets were created by combining the clover and rose pollen as follows: (1) 100% clover to 0% rose, (2) 75% clover to 25% rose, (3) 50% clover to 50% rose, (4) 25% clover to 75% rose, and (5) 100% rose to 0% clover. To each artificial diet, 15 ml of sugar water was added (1:1 sugar to water) to form a cohesive patty. All artificial pollen diets were stored at 4 °C. Smaller pollen balls were then formed from the larger pollen patties. Controls were removed and then returned to natural pollen ball. Pollen balls for each diet and controls were weighed using a Mettler analytical balance (accuracy 0.01 mg) and eggs or first stage larvae were placed randomly on one of the five artificial pollen balls or controls. After bees had fully developed, the remaining pollen was collected, re-weighed using a Mettler analytical balance (accuracy 0.01 mg), and stored at −80 °C for

preservation. The total amount of pollen consumed, not the starting pollen amount, was used in the following analyses.

Macronutrient profile of artificial pollen diets

Protein, sugar, amino acids, and fatty acids from the pure 100% clover diet and the 100% rose diet were measured. Specifically, to obtain a dry weight, pollen-masses were placed in a drying oven at 80 °C for 48 h. Samples were then divided in half with a sterile blade and reweighed for sugar and protein analysis, respectively. Samples for sugar analysis were suspended in 200 µl of distilled water and stored at −80 °C until analysis. Following previous methods from Kapheim *et al.* (2011) and Richards and Packer (1994), sugar samples were thawed in a 50 °C water bath for 3 min and then centrifuged at >2500 rpms for 5 min. Sugar content was measured in degrees Brix by placing 20 µl of the supernatant on a handheld refractometer. Based on the weight of the original pollen ball, we determined the milligrams of sugar in each sample.

To measure protein content in the pollen samples, we followed methods described in Kapheim *et al.* (2011). Previously dried and weighed pollen ball samples were sent for nitrogen (N) analysis at Brookside Laboratories, Inc. (New Knoxville, Ohio, U.S.A.). Using combustion and thermal conductivity detection in a Carl–Erba total nitrogen analyser (AOAC International 1996; Gavlak *et al.*, 2003), percent crude protein was calculated by multiplying the %N of the sample by the standard conversion factor of 6.25%. The total protein in the sample was calculated as the percent crude protein times the dry mass of the sample divided by 100.

Metabolite profiling of pure 100% clover and 100% rose pollen was conducted at the Metabolomics Core of the Mayo Clinic (Rochester, Minnesota, U.S.A.). A total of 10 mg of pollen was submitted for an amino acid profile to determine the concentration (nmols) of 42 amino acids and 40 mg of pollen was submitted for a NEFA (Non-Esterified Fatty Acid) panel to determine the concentration (nmols) of 12 fatty acids.

Statistical analysis

Head width of the adult bees, survivorship, protein, sugar, fatty acid, and amino acid amounts were all separately tested with the Shapiro–Wilk test for normality and Levene's test for equality of variance. Head width for females was analysed using a two-factor ANOVA followed by a post-hoc Wilcoxon signed-rank tests for pairwise comparisons. Head width for males was not normally distributed and was compared with a Kruskal-Wallis test followed by post-hoc Dunn's test for pairwise comparisons. Similarly, total development time was not normally distributed, and thus compared with a Kruskal-Wallis test followed by post-hoc Dunn's test for pairwise comparisons (Sokal & Rohlf, 1995). Survival data were analysed using Kaplan-Meier estimates and survival rate by the group was compared using a log-rank test. All statistical analyses were performed in JMP v.12 (SAS, Cary, North Carolina, U.S.A.). All reported *P*-values are two-tailed.

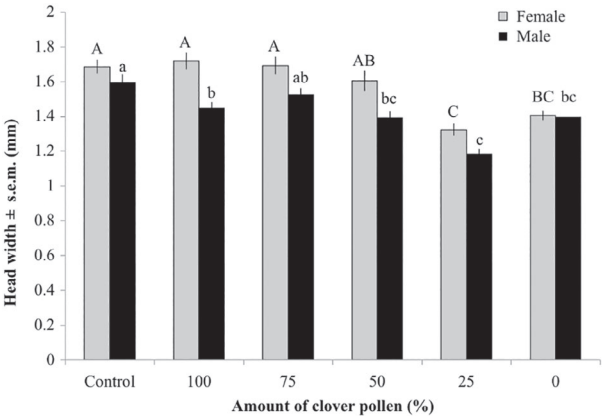

Fig. 2. The average head width (± standard error) of *C. calcarata* females (grey) and males (black) raised on diets containing differing ratios of clover to rose pollen. The average head width of both females and males decreased as the overall amount of clover in the diet decreased. Upper case letters indicate significant differences in female head width ($P < 0.05$). Lower case letters indicate significant differences in male head width ($P < 0.05$).

Results

Artificial pollen diets

To determine the effects of different pollen ratios on growth and development of the native bee, *C. calcarata*, we compared adult head width, survivorship, and developmental rates of bees raised on five different artificial diets compared to controls raised on natural pollen balls. Adult head width and sex were both significantly different across the six diets with varying ratios of white clover to rose pollen (Fig. 2; two-factor ANOVA diet: $F = 8.28$, $df = 5$, $P < 0.0001$; sex: $F = 17.56$, $df = 1$, $P < 0.0001$; sex*diet: $F = 0.99$, $df = 5$, $P = 0.42$). Females raised on diets that contained more clover pollen were significantly larger than those raised on diets containing less clover and more rose pollen (Fig. 2; $N = 80$; Kruskal-Wallis $\chi^2 = 15.44$; $df = 5$; $P = 0.009$). Specifically, female *C. calcarata* raised on controls of natural pollen balls or 75% clover pollen was significantly larger than bees raised on either 25% clover pollen or 0% clover pollen (Fig. 2).

Male bees raised on natural pollen balls were significantly larger than males in all treatment groups, except bees raised on 75% clover pollen balls (Fig. 2; $N = 59$; Kruskal-Wallis $\chi^2 = 21.90$; $df = 5$; $P = 0.0005$). When comparing males across treatment groups, we found a similar trend to the females where *C. calcarata* raised on diets that contained more clover were significantly larger than those raised with less clover and more rose (Fig. 2).

In addition to significant effects on adult head width, artificial diets had significant effects on the development rate. Overall, bees raised on diets containing less clover and more rose pollen took significantly longer to develop (Fig. 3; $N = 230$; Kruskal-Wallis test $X^2 = 40.30$, $df = 5$, $P < 0.0001$). The total development time for control bees raised on natural pollen balls was 33.15 ± 0.14 days (mean ± s.e.m.), which was significantly shorter than bees raised on either 0% clover

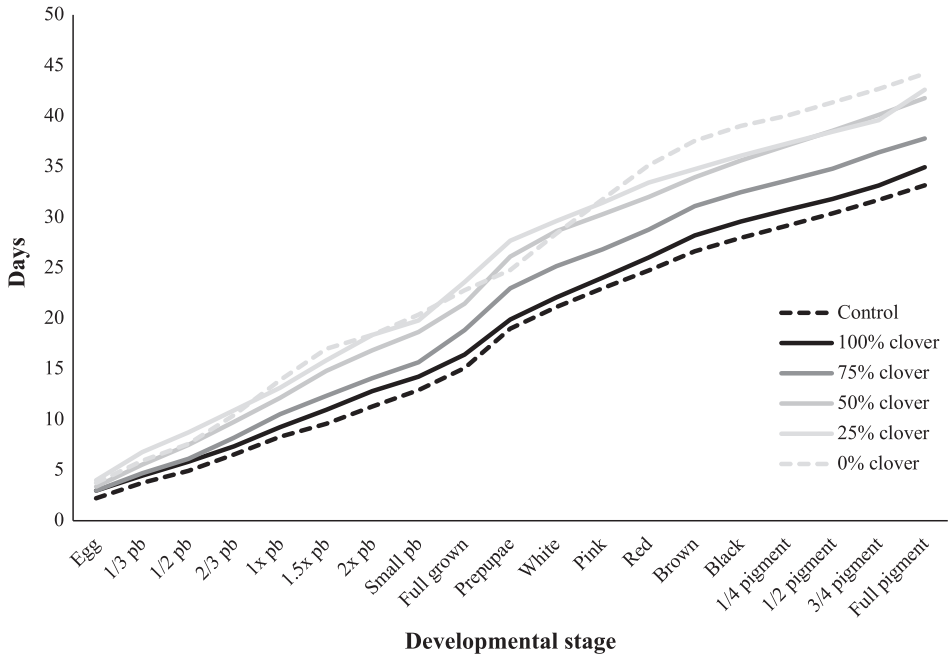

Fig. 3. The cumulative development rate of *C. calcarata* raised on five artificial diets with varying ratios of clover to rose pollen versus controls raised on natural pollen balls (dashed line). *Ceratina calcarata* raised on diets containing less clover pollen and more rose pollen took significantly longer to develop ($P < 0.05$). Developmental stages taken from Rehan and Richards (2010a).

(44.10 ± 0.45 days) or 25% clover (42.60 ± 0.34 days), but was not significantly different from those raised on 100% clover (34.92 ± 0.18 days), 75% clover (37.76 ± 0.20 days), or 50% clover (41.78 ± 0.22 days; Table S1). Overall, total development time increased as the percentage of clover decreased in the diet. Most of this increase in development time was noted in the later larval stages when offspring feeding was complete (Table S1).

More bees survived on a natural diet compared to either artificial diet (Fig. 4; Log-rank test; $X^2 = 56.81$, $P < 0.0001$). Between treatment groups, bees raised on 100% clover pollen had a significantly higher survival rate than those raised on 25% clover and 0% clover. Those raised on 0% clover had significantly higher mortality than 50% clover or 75% clover (Fig. 4).

Macronutrient profile of artificial pollen diets

To further investigate the nutritional differences between clover and rose pollen, we compared the macronutrient profile of 100% clover pollen versus 100% rose pollen. Pure rose pollen contained significantly more sugar than clover pollen (Table 1). There were no significant differences in protein concentration between rose and clover pollens (Table 1). Of the 42 amino acids sampled, clover pollen contained higher concentrations of 29 of the amino acids sampled, while rose pollen contained only two amino acids at higher concentrations (Table S2). Specifically, clover pollen contained higher concentrations of all the essential amino acids, including significantly more valine, leucine, isoleucine, phenylalanine, lysine, and tryptophan (Table 1). Of the 12 fatty acids tested, rose pollen contained higher concentrations of seven of the fatty acids, including significantly more myristic, palmitoleic, and linolenic (Table 1). Overall, clover pollen contained significantly higher concentrations of all the essential amino acids, and lower amounts of sugar, protein, and fatty acids.

Discussion

Ceratina calcarata raised on diets consisting of more clover pollen and less rose pollen had more survivors, who developed into larger adults with shorter developmental times. Although clover pollen had lower concentrations of sugar and fatty acids, clover pollen had higher concentrations of all essential amino acids. Bees raised on control diets containing polyfloral pollens were significantly larger and had higher survivorship than any treatment group, including the monofloral artificial pollen diets of rose or clover and pollen diets containing both pollens. Previous research has shown *C. calcarata* forage from over 27 plant families throughout the foraging season and the average protein in each pollen ball ranges from 4 to 10 mg (Lawson *et al.*, 2016). More research is needed to characterise the remaining macronutrient profile of natural *C. calcarata* pollen balls, as well as, how specific macronutrients effect individual foraging preferences. Previous research in honey bees has shown that nurse bees fed on polyfloral blends lived longer following parasitism than nurse bees fed on monofloral blend (Pasquale *et al.*, 2013). Similarly, polyfloral diets have been experimental shown to increase the survival of solitary bees (*Osmia corunta*; Eckhardt *et al.*, 2014). More importantly than pollen diversity, increased protein-rich pollen quality in honey bees is associated with increased tolerance to parasitism (Pasquale *et al.*, 2013)

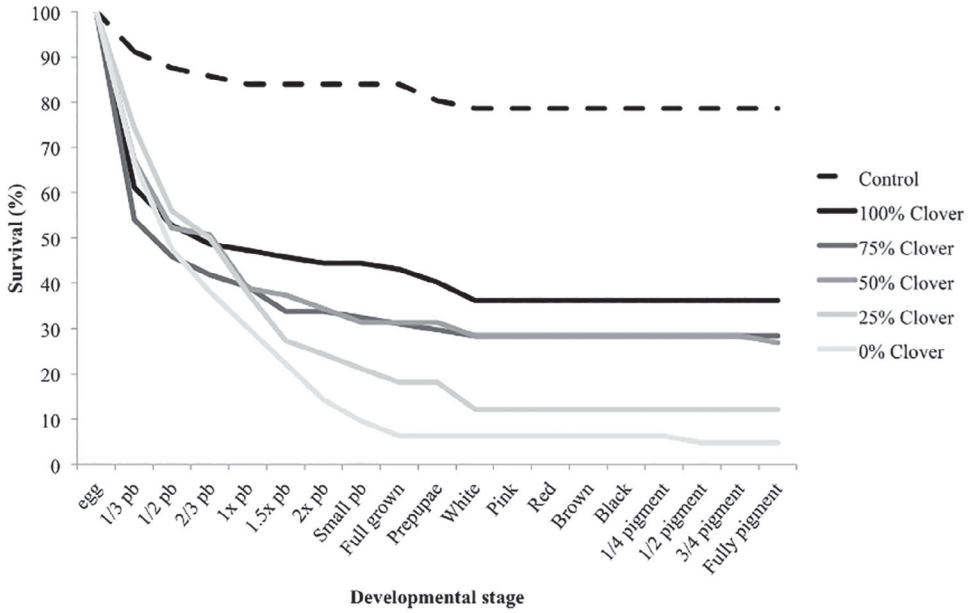

Fig. 4. Percent survival of *C. calcarata* raised on artificial pollen diets varying in the ratio of clover to rose pollen compared to controls raised on natural pollen balls. Larval stages range from egg to pre-pupae and pupal stage from white to fully pigmented. Mortality for bees raised on artificial diets was highest in the early stages of development with a plateau occurring once the pupal stage was reached. Overall, bees raised on diets containing more rose pollen had significantly higher rates of mortality than those raised on diets of clover or controls ($P < 0.05$).

Table 1. Macronutrient profile of 100% clover pollen versus 100% rose pollen, including raw sugar, protein, essential amino acids, and fatty acids.

Group	Macronutrient	Clover	Rose	*t*-ratio	*P*-value
Sugar (mg)	Sugar	3.96	11.44	7.95	**0.02**
Protein (mg)	Protein	15.16	19.99	1.36	0.28
Amino acid (nmol/10 mg)	Threonine	0.82	0.27	−11.00	**0.0082**
	Valine	1.07	0.49	−25.94	**0.0015**
	Methionine	0.20	0.11	−3.18	0.09
	Leucine	1.20	0.37	−10.21	**0.0095**
	Isoleucine	0.41	0.23	−7.06	**0.0195**
	Phenylalanine	1.24	0.54	−6.94	**0.0201**
	Lysine	1.71	0.14	−5.11	**0.0037**
	Histidine	1.67	0.25	−1.74	0.22
	Arginine	0.50	0.25	−3.42	0.08
	Tryptophan	0.21	0.03	−11.67	**0.0073**
Fatty acid (nmol/40 mg)	Linolenic*	147.67	199.53	4.87	**0.00397**
	Myristic	0.46	2.36	36.30	**0.0008**
	Palmitoleic	0.32	1.11	8.20	**0.0146**
	Arachidonic	0	0	n/a	n/a
	Palmitelaidic	0	0	n/a	n/a
	Linoleic	18.71	30.24	1.55	0.26
	Palmitic*	85.69	163.42	0.89	0.47
	Oleic	10.41	15.12	0.51	0.66

Bold numbers indicate a significant difference ($P < 0.05$). Asterisks indicate readings were over standard curve.

and less sensitivity to pesticides (Wahl & Kurt, 1983). More nutritious pollen diets, including more essential amino acids, generally, have been shown to affect pathogen resistance and stress in honey bees (reviewed in Huang, 2012).

We found that *C. calcarata* raised on clover pollen had increased survivorship and became larger adults than bees raised on rose pollen (Figs 1–3). Previous research found a range of protein percentages for pollen from the Rosaceae family from 28% to 45% using the Micro-Kjeldahl analysis technique compared to our results of 22% protein or 20 mg per sample (summarised in Roulston *et al.*, 2000). Previous research only sampled one species from the same genus, *Rosa woodsii* Lindl., which contained 44.5% protein (summarised in Roulston *et al.*, 2000). *Trifolium* spp. ranged from 13% to 35% protein

compared to our results of 23% protein or 15 mg per sample (summarised in Roulston *et al.*, 2000). These results indicate that even within closely related species, there is a wide range of protein content. Both white clover and rose pollen in our study were found to have similar levels of protein. In *Lasioglossum zephyrum*, pollen protein ranging from 20% to 37% protein was found to be correlated to adult body size (Roulston & Cane, 2002). In addition, pollen protein is associated with bee visitation to flowers in multiple species of bees, including bumble bees, honey bees, and male/cleptoparasitic bees (Russo *et al.*, 2019). Manipulative research has shown that bumble bees forage for specific 5:1 protein to lipid ratios (Rasheed & Harder, 1997, Vaudo *et al.*, 2016a,b). This implies that although the clover pollen contained less protein than the rose pollen, possibly the protein to lipid ratio is more important to growth and development than protein content alone.

Carbohydrates play an important role in pollen nutrition. Bees use carbohydrates for energy and bees have been shown to provision themselves with sugar before foraging flights (Brodschneider & Crailsheim, 2010). Adult honey bees have been shown to need 4 mg of sugars per day for survival (Barker & Lehner, 1974). Honey bee foragers have been shown to prioritise carbohydrate dietary requirements over essential amino acids (Paoli *et al.*, 2014), although adult honey bees receive most of their sugar intake through nectar, not pollen. We found that rose pollen contained almost 4× the amount of sugars as clover pollen (Table 1). The nutritional requirements of the small carpenter bee likely differ throughout its life and may be different from the honey bee, so carbohydrates may not be the limiting resource for healthy growth and development, instead, other macronutrients like amino or fatty acids could play essential roles. In addition, we need more information about how the small carpenter bees obtain and digest their carbohydrates.

In addition to protein and carbohydrates, a complete diet includes amino and fatty acids. Lipids are important in healthy honey bee development, nutrition, and growth and are involved in the synthesis of fat, glycogen, and cell membranes (Graham, 1992; Manning, 2001). Colonies fed with increasing levels of lipids have been shown to have large colony sizes (Herbert *et al.*, 1980). Bumble bees have been shown to survive best on a 10:1 protein to lipid diet, but forage at a ratio closer to 5:1 (Vaudo *et al.*, 2016a). Pollens high in oleic and palmitic acids have been hypothesised to play the greatest role in overall honey bee nutrition (Manning, 2001). We found that both clover and rose pollen contained high levels of both oleic and palmitic acids.

Amino acids are important to the development of *Ceratina calcarata* as demonstrated by the increased survival rate of white clover pollen. The main observed difference between the clover and rose pollen was clover pollen was more similar to the ideal amino acid ratio for growth and development in honey bees (Table 2; De Groot, 1953). Honey bees have been shown to select pollen containing more essential free amino acids (Cook *et al.*, 2003). In addition, previous research has shown that a lack of essential amino acids in both honey bee (Bonoan *et al.*, 2019) and bumble bee (Moerman *et al.*, 2016) colonies slows colony growth.

Table 2. Ideal amino acid ratio for bee health according to De Groot (1953) compared to the amino acid ratio in clover and rose pollen.

	Ideal ratio	Clover	Rose
Threonine	3	3.90	9.00
Valine	4	5.10	16.33
Methionine	1.5	0.95	3.67
Leucine	4.5	5.71	12.33
Iso-leucine	4	1.95	7.67
Phenylalanine	2.5	5.90	18.00
Lysine	3	8.14	4.67
Histidine	1.5	7.95	8.33
Arginine	3	2.38	8.33
Tryptophan	1	1.00	1.00

Clover pollen matches more closely to the ideal ratio of macronutrients than rose pollen.

Conclusion

Ceratina calcarata raised on artificial pollen diets containing more clover pollen were larger and healthier overall suggesting that for this cosmopolitan native pollinator species the correct essential amino acid profile may be the limiting nutritional factor in their diet. More importantly, *C. calcarata* raised on multifloral control diets had significantly higher survival and growth indicating that a diverse diet is needed for healthy development. Previous research has shown that *C. calcarata* are generalist foragers and change foraging preferences throughout the season (Lawson *et al.*, 2016). In intensified agricultural lands, *C. calcarata* have been shown to have decreased fitness, likely due to parasite load and nutritional deprivation (Nooten & Rehan, 2019). Nutritional deprivation of the eldest daughter leads to smaller, more submissive adults (Lawson *et al.*, 2017). Changes in floral availability and any reduction in floral diversity are therefore likely to have negative effects on not only the body size but also survivorship of this species. More research is needed to fully understand the foraging preferences and nutritional requirements of *C. calcarata* and other wild bees, but one small and easy remedy would be to increase a variety of pollen sources, including clover availability to help increase the survival and health of native bees in the wild.

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Author contributions

SPL was involved with the experimental design, set-up, data analysis, and writing of the manuscript. KBK assisted with data collection, data analysis, and writing of the manuscript. SMR contributed to the designing of the experiment, data analysis, and writing of the manuscript. All authors edited the manuscript and approved it.

Data availability statement

Data archival location: Figshare https://figshare.com/projects/Pollen_composition_significantly_impacts_development_and_survival_of_the_native_small_carpenter_bee_Ceratina_calcarata/89129

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. The development rate of *C. calcarata* raised on five artificial diets with varying amounts of clover and rose pollen versus controls raised on natural pollen balls. Bold numbers indicate a significant difference in development time compared to controls ($P < 0.05$). *Ceratina calcarata* raised on diets with more rose pollen took significantly longer to develop.

Table S2. Complete macronutrient profile of 100% clover pollen versus 100% rose pollen, including raw sugar, protein, all measured amino acids and fatty acids, and standard error. Bold numbers indicate a significant difference ($P < 0.05$). Asterisks indicate readings were over the standard curve.

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