Differential reward in “male” versus “female” pollen of functionally dioecious Solanum

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Abstract

Premise: Five to six percent of angiosperm species exhibit a dioecious sexual system, with unisexual “male” or “female” flowers borne on separate plants. The consequent need for inter-individual pollen exchange is a special challenge for taxa where pollen is the sole pollinator reward. Dioecious Australian Solanum assure visits from pollen-foraging bees via production of inaperturate pollen in functionally female (morphologically bisexual) flowers. Biochemical composition of pollen from Australian Solanum has not been assessed nor compared to porate pollen from staminate flowers to reveal whether these flowers differ in their pollinator reward potential.

Methods: Porate pollen from male flowers and inaperturate pollen from functionally female flowers of two functionally dioecious Australian species were compared for protein and amino acid content. We also assessed pollen from bisexual and staminate flowers of a closely related andromonoecious species, in which all pollen is porate, as a comparison across co-occurring sexual systems.

Results: In both functionally dioecious species, porate pollen grains from staminate flowers had significantly higher levels of proteins and amino acids than inaperturate pollen grains from functionally female flowers. Levels of proteins and amino acids were highest in bisexual and staminate flowers of the andromonoecious species.

Conclusions: Higher levels of proteins and amino acids in porate pollen of “male” flowers in our functionally dioecious Solanum species suggests a greater nutritive reward for bees foraging on “male” plants than for those foraging on functionally “female” plants. Greater reward in porate pollen (including andromonoecious species) may be connected to the potential to generate a pollen tube.

Keywords: andromonoecy, bee pollination, bicinchoninic acid assay, buzz pollination, dioecy, HPLC, Leptostemonum, pollen protein, pollination, Solanaceae

The majority of angiosperm species (~94%) exhibit some form of co-sexuality, with at least some plants bearing bisexual flowers (Renner and Ricklefs, 1995; Renner, 2014)—perhaps because of an increased assurance of reproductive success even in the face of a potentially increased risk of inbreeding (Bateman, 1952). The remaining 5–6% of the species exhibit a dioecious sexual system (Renner and Ricklefs, 1995; Renner, 2014), where unisexual (staminate or carpellate) flowers are found on separate plants and the potential for inbreeding through selfing (in self-compatible taxa) is avoided but inter-individual pollen exchange is required to ensure reproductive success (Charlesworth and Charlesworth, 1978). In the genus Solanum (Solanaceae), dioecy is rare, having been observed in just 19 (~1%) of the ~1400 species in the genus, but it has independently evolved perhaps as
many as eight separate times (6 times in the New World Tropics and 1–2 times in Australia) (Anderson and Symon, 1989; Knapp et al., 1998; Anderson et al., 2015; Martine et al., 2019). In each of these instances, the sexual system presents as morphologically androdioecious, with only bisexual flowers on some individual plants and only stamine flowers on others. What is true by the naked eye is quite something else in terms of functionality, however, because the morphologically bisexual flowers bear anthers that are either free of pollen or that produce inaperturate pollen (Anderson and Gensel, 1976; Anderson, 1979; Knapp et al., 1998; Anderson et al., 2015) (Figure 1). Bisexual flowers that produce no or inaperturate pollen are thus functionally carpellate, with morphologically (and functionally) stamine flowers producing porate pollen, meaning that the sexual system is more appropriately identified as functionally dioecious. Given that androdioecy is predicted to be an exceedingly rare evolutionarily unstable state among plants and a likely transition from hermaphroditism to “full” dioecy (Charlesworth, 1984; Pannell, 2002), these Solanum species are seemingly mid-transition somewhere between androdioecy and dioecy.

Retention of stamens and/or “nonfunctional” inaperturate pollen in functionally female flowers may be correlated with the buzz pollination syndrome (also known as sonication) present in nearly all Solanum species and nearly 200 other genera (Vogel, 1978; Buchmann, 1986). In buzz-pollinated Solanum flowers, nectar is absent, pollen is the sole floral reward, and anthers typically serve as both pollinator attraction and landing platforms for foraging (Anderson and Symon, 1988, 1989; Connolly and Anderson, 2003; Anderson et al., 2015). The loss of stamens (and pollen) might thus be untenable as pollinator visits would decrease or cease to occur given the reported ability of pollen-foraging bees to assess the presence of anthers and pollen before visiting a flower (Zimmerman, 1982; Creswell and Robertson, 1994; Connolly and Anderson, 2003; Ashman et al., 2005).

For this study, we worked with a set of closely related Solanum species of the S. dioicum Group (Bean, 2004; Martine et al., 2009, 2019), a clade of “spiny solanum” (Solanum subgenus Leptostemonum) species native to the Australian Monsoon Tropics with the greatest known richness of functionally dioecious species (13 currently described and two forthcoming) in the genus (Anderson

![FIGURE 1](pollen.png)

**FIGURE 1** Pollen and flower types for *S. ossicruentum*, one of the two functionally dioecious Solanum species in Australia studied. (A) Porate pollen (above) produced by anthers of “male” flowers (below); note multiple flowers per inflorescence in image below. (B) Inaperturate pollen (above) produced by functionally “female” (but morphologically bisexual) flowers (below); note flower borne solitarily in image below. SEM images by Alice Butler (previously published by Martine et al. [2016]); flower images by C. Martine
et al., 2015; Martine et al., 2016, 2019; T. M. Williams, Bucknell University; and C. T. Martine, unpublished manuscript). Previous work done in this group found that porate “male pollen” is produced in greater quantity per flower and is both larger and more stainable with aniline blue in lactophenol than inaperturate “female pollen” (Anderson and Symon, 1989), but a comparison of their biochemical composition as it relates to potential differences in the rewards available for floral visitors has not been done.

Greater understanding of differences in pollen rewards should inform questions related to the maintenance of the functionally dioecious sexual system, where pollinator visits to functionally female flowers might depend on provision of rewards equal to or greater than those provided by staminate flowers (Martine and Anderson, 2007), especially in populations of plants in which differences in inflorescence architecture (functional females borne solitarily and staminate flowers borne in many-flowered cyms) lead to many more staminate flowers being available than female flowers (Anderson and Symon, 1989). As an example, S. asymbriphyllum may bear as many as 80 male flowers per inflorescence; male plants might then have on the order of 1000 male flowers for every 15–20 flowers produced by a female plant (C. T. Martine, personal observations). Given that directional flow of porate pollen from male plants to the stigmas of female plants is required for seed production (Anderson and Symon, 1989) and population sustainability, one might wonder why pollen foraging bees would leave a given heavily provisioned male plant to forage on a female plant bearing fewer flowers. Is it possible that inaperturate “female pollen” offers an incentive that inspires such movement?

Because the diets of most bees consist largely of floral rewards, the primary protein resource for both adults and larvae is pollen (Michener, 2000) and “bee pollen” (pollen foraged by bees) contains their primary source of essential amino acids (de Groot, 1953). Although honey bees are not buzz pollinators, it has been shown that the gustatory sensilla on their mouth parts are diagnostically responsive to certain proteins and amino acids (Whitehead and Larsen, 1976; de Brito Sanchez, 2011). Bees are also known to adjust their foraging behavior depending on the protein concentrations of pollen produced by flowers they visit (Roulston et al., 2000), so the total amino acid and protein content of pollen is of special interest. While we know little about the behavior of the species of Amegilla (blue-banded bees), Nomia (sweat bees), and Xylocopa (carpenter bees) that act as the primary buzz pollinators of the Solanum species in our study group (Anderson and Symon, 1988), much has been learned about Bombus (bumblebees), in part because of their role as a key component of Solanum agriculture (e.g., tomatoes, eggplants). Bumblebees not only can assess the presence/absence of pollen encountered while sonicating (Buchmann and Cane, 1989), but they also might detect and learn the signatures of particular volatile compounds to detect the presence of pollen—although there is still a large gap in our knowledge regarding what pollen bees choose and how they come to that decision (see Dobson et al., 1999; Robertson et al., 1999; Schmidt and Hanna, 2006; Hanley et al., 2008; Kitaoka and Nieh, 2009; Leonhardt and Blüthgen, 2012). On the basis of previous theoretical assumptions (e.g., Martine and Anderson, 2007), higher reward levels in the inaperturate pollen of morphologically female flowers have been predicted to be one way that pollen foragers are driven to repeatedly visit functionally female plants.

To measure potential differences between porate and inaperturate pollen types, we sought to determine quantity and quality of proteins and free amino acids in the pollen of our study taxa. We tested the hypothesis that protein and amino acid composition differs between porate and inaperturate pollen grains produced in, respectively, staminate and morphologically bisexual flowers of two functionally dioecious Australian Solanum species (S. ossicatum Martine & J. Cantley [Martine et al., 2016] [Figure 1] and S. sejunctum K. Brennan, Martine, & Symon [Brennan et al., 2006]). Because pollen from both the bisexual and staminate flowers of andronomonoecious congeners is porate, we also assessed and compared the pollen of a closely related sympatric andromonoecious species (S. ultraspinosum A.R. Bean [Bean, 2016]).

In some ways our study, resembles that of Buchmann (1986), who looked at differences in pollen chemistry across 17 Solanum species. Among those was S. appendiculatum—a functionally dioecious taxon that is distantly related to our study lineage and represents an independent New World origin of functional dioecy (Anderson, 1979). Although Buchmann anticipated stark differences between porate “male pollen” and inaperturate “female pollen”, the two morphs were found to be roughly equivalent in nitrogen content (a proxy for amino acid and protein content, which were not directly measured) and caloric value. Since Buchmann’s extensive review of Solanum pollen the ability to measure protein and amino acid content in biological samples has significantly improved. While Buchmann utilized nitrogen content as a proxy, our study utilizes methodology established by Vanderplanck et al. (2014) which compares the nitrogen approximation method of protein quantification with a combination of bicinchoninic acid assay BCA total protein assay and HPLC for protein and amino acid analysis of pollen. Our study represents the first quantification of amino acids and proteins in Australian Solanum and is the first to directly test for these components in pollen produced by any functionally dioecious Solanum species.

MATERIALS AND METHODS

Pollen collection and storage

Pollen samples were collected from plants cultivated in the David Burpee research greenhouse at Bucknell University
(Lewisburg, PA, USA) using plant-care protocols described by Hayes et al. (2019). Pollen was collected from S. sejunctum, S. oisciruentum, and S. ultraspinosum from freshly opened flowers utilizing either a probe or a headless electric toothbrush. Pollen from multiple flowers and plants of the same morphology and species was pooled until 8 mg dry matter was acquired to run the assays described below. This pooling strategy was developed to accommodate varying pollen yields. Porate pollen was more abundant (due to greater staminate flower production) than inaperturate, and environmental factors such as time of day, humidity, and temperature impacted pollen yield. Approximately 1500 flowers were utilized per species to provide sufficient pollen for protocol optimization and replicates for assays performed. Inaperturate and porate pollen were only compared from the same collection days to limit the impact of environmental variables. Because temperature is an important environmental factor affecting the chemical stability of stored pollen (Stanley and Linskens, 1974), pollen was stored immediately after collection at −80°C to avoid protein degradation.

Protein analysis

Pollen was processed and proteins extracted using a protocol adapted from Hurkman and Tanaka (1986) and Kalinowski et al. (2001). For each extraction, 8 mg of dry mass pollen was lysed with a dounce homogenizer with 400 µL of extraction buffer (500 mM Tris-hydrochloride, 50 mM EDTA, 700 mM sucrose, 2% v/v 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 100 mM KCI). The homogenized extract was poured into a 1.5 mL microcentrifuge tube and incubated while shaking for 15 min at 4°C after which 500 µL of water-saturated phenol was added to the sample. After a short incubation at room temperature with agitation, the phenolic phase was recovered by centrifugation at 8000 rpm for 10 min at 4°C. Extraction buffer was added to the sample, and after vortexing and incubation for 3 min at room temperature under agitation, the phenolic phase was recovered again by centrifugation at 8000 rpm for 10 min at 4°C. The upper phenol phase (100 µL) was harvested, and 400 µL of ammonium acetate methanol was added to precipitate the solution overnight at −20°C. Precipitated proteins were then recovered by centrifugation at 8000 rpm at 4°C for 15 min. Precipitates were dried under vacuum (4 h minimum) in a speedvac set to high speed without heat.

The dry pellet was stored at −80°C and resuspended in a solubilization buffer containing 8 M urea, 4% v/v 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), and 0.2% w/v carrier ampholytes for later use in protein quantification. Total proteins were quantified using the Pierce BCA Protein Assay Kit and NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA).

Hydrolyzed amino acid analysis

A hydrolysis solution (1 mL of 6 N HCl, 0.1% v/v phenol, and 500 µM norleucine) was added to 5 mg of pollen in each tube (per Vanderplanck et al., 2014). The samples were then frozen using liquid nitrogen for 1 min before incubation at 110°C for 24 h while shaking at 800 rpm (benchtop orbital shaker) to avoid methionine degradation. Afterward, the hydrolysate was evaporated under a vacuum in a boiling bath at 100°C, then 1 mL of sodium citrate buffer, pH 2.2, was added to each tube. Samples were mixed, filtered (0.2 µm), aliquoted in HPLC, and sent for ultra performance liquid chromatography (UPLC) and mass spectrometry (MS) analysis at the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, Missouri, USA) via Science Exchange. These methods allow us to identify the different amino acids present in our samples. For the technical replicate analyses, the derivatization step was performed twice on the same samples.

Free amino acid analysis

Approximately 30 mg of pollen was mixed with 200 µL of extraction solution (1 mM norleucine, 0.1 N HCl, and 2% thioglycol) in an ultrasonic bath for 30 min. Then, 100 µL of 15% dihydrated 5-sulfosalicylic acid was added, and samples were incubated at room temperature for an additional 5 min to precipitate proteins. The precipitate was then centrifuged (8120 × g) at room temperature for 5 min. The supernatant (250 µL) was filtered (0.22 µm) and centrifuged at 6710 × g for 10 min at room temperature, 100 µL of a pH adjustment solution (1:1, 1.5 N NaOH and pH 2.2 buffer) was added to the supernatant. The samples were then dried under vacuum (4 h minimum) in a speedvac set to high speed without heat and resuspended in 20 mM HCl. During derivatization, 10 µL was used for the reaction with a volume totaling 100 µL; 1 µL of the reaction was injected into the UPLC system. UPLC was coupled with mass spectrometry (MS) to identify the different amino acids in our samples at the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO, USA) via Science Exchange. For the technical replicate analyses, the derivatization step was performed twice on the same samples.

Statistical analyses

Data for each species were analyzed using independent t tests in SPSS version 26 (IBM, Armonk, NY, USA) to test the null hypothesis of no difference in protein contents between inaperturate and porate pollen types of the same species. The same statistical analysis was applied to protein content, hydrolyzed amino acid content, and free amino acid content. For analysis of essential and non-essential amino acids, each amino acid was compared between the pollen types within species.
RESULTS

Total protein analysis

A significant difference in protein concentration was found between the porate (male) and inaperturate (female) pollen of the two functionally dioecious species (independent t-test, $t = 9.9146$, df = 123, $P < 0.001$ for $S. sejunctum$, $t = 11.3$, df = 123, $P < 0.001$ for $S. ossicruentum$) (Figure 2). On average, porate pollen contained 10–15% more protein than in the inaperturate pollen from the same species. Protein concentrations of 1668.5 ± 65.0 μg/mL in porate and 1427.4 ± 38.4 μg/mL in inaperturate pollen were recorded for $S. sejunctum$ and concentrations of 1609.5 ± 34.4 μg/mL in porate and 1468.2 ± 34.4 μg/mL in inaperturate pollen were recorded for $S. ossicruentum$, indicating a significant difference between the mean protein concentration in porate pollen than in inaperturate pollen in both species (independent t-test, $t = 9.9146$, df = 123, $P < 0.001$ for $S. sejunctum$, $t = 11.3$, df = 123, $P < 0.001$ for $S. ossicruentum$).

For the andromonoecious taxon, $S. ultraspinosum$, both flower types produce porate pollen—with each plant thus bearing a combination of functionally male flowers and functionally bisexual flowers. Surprisingly, for pollen produced by bisexual flowers, the mean concentration (2398.6 ± 65.0 μg/mL) was significantly higher than in pollen from staminate flowers (1466.4 ± 27.0 μg/mL) (independent t-test, $t = 43.92$, df = 123, $P < 0.001$). To allow comparison with previously published results, concentrations were converted to percentage protein per gram of dry matter. These results can be seen in Appendix S1.

Free amino acid analysis

Hydrolyzed amino acid measurements include both free amino acids and those bound into peptides and proteins (Table 1). Sixteen amino acids were present at detectable levels in our samples. The results for phenylalanine are not presented as the retention time peak for this amino acid overlaps with that of norleucine, the internal standard.

Amino acid components varied between porate and inaperturate pollen from functionally dioecious species. Porate pollen had higher levels of amino acids when compared to inaperturate pollen of the same species (independent t-test, $t = 6.796$, df = 4, $P = 0.0024$ for $S. sejunctum$; $t = 3.4988$, df = 4, $P = 0.0249$ for $S. ossicruentum$). This trend held true for both essential and non-essential amino acids.

In $S. ultraspinosum$, the andromonoecious species, pollen protein content varied between the two porate pollen types. Pollen from the bisexual flowers is significantly higher in all individual amino acids than in the staminate flowers (independent t-test $t = 7.9178$, df = 4, $P = 0.0014$). The highest concentrations of individual non-essential amino acids were for aspartic acid (19.88 ± 0.715 mg/g dry matter), glutamate (18.82 ± 0.649 mg/g dry matter), and proline (15.72 ± 0.320 mg/g dry matter) all from pollen harvested from bisexual flowers.

Across all three species studied, the essential amino acid with the lowest concentration was methionine, and the highest was leucine. The non-essential amino acid with the lowest concentration was cysteine; the most abundant was proline, which had the highest levels among all the essential and non-essential amino acids.
(8 essential amino acids, 2 modified amino acids, 8 non-
essential amino acids) were detected in free form in the
pollen we tested. Norleucine was excluded from our analysis
because of its use as an internal standard.

Six of the nine free essential amino acids were found
across all the species: tryptophan, leucine, isoleucine, valine,
threonine, and lysine. Tyrosine was present only in trace
amounts in porate pollen from the functionally dioecious
species and in the bisexual flowers of S. ultraspinosum. Free
histidine was only present in porate pollen of bisexual
flowers of S. ultraspinosum and inaperturate pollen from
S. ossicruentum. Free arginine (which had a high total
concentration; Table 1) was totally absent in all the samples.
Methionine was present in trace amounts in porate pollen
from S. ossicruentum (0.041 ± 0.001 ng/100 g) and from
hermaphrodite flowers of S. ultraspinosum (0.055 ± 0.002)
ng/100 g).

Eight of the 10 free amino acids were found in varying
levels among all pollen samples. Proline is the most abun-
dant free amino acid in the pollen of all the samples, with
the porate pollen having an average of 10% higher content
than the inaperturate pollen in the functionally dioecious
species (Table 2). Cysteine and glutamine are absent in all
pollen samples. The derivative amino acid GABA, a
neurotransmitter in bees (Kiya and Kubo, 2010), was also
found in all pollen samples. GABA is significantly higher in
inaperturate pollen grains from functionally female flowers
of S. sejunctum (0.141 ± 0.011 ng/100 g, independent t-test,
t = 4.8623, df = 4, P = 0.083) and porate pollen from bisexual
flowers of S. ultraspinosum (0.789 ± 0.040 ng/100 g, in-
dependent t-test, t = 10.2092, df = 4, P = 0.005) (Table 2).

### Analysis of nutritive value of pollen

Evaluating the amino acid content as a measure of total
amino acid content per 100 g of dry matter (TAA) and the
total essential amino acid content per 100 g of dry matter
(TEA) enables an evaluation of the nutritive value of a food
substance (Oser, 1959; Biel, 2009). The amino acid com-
positions of porate pollen from S. ultraspinosum her-

maphrodite flowers had the highest average values (g/100 g
dry mass [DM]) of total amino acids (TAA = 17.18 g/100 g
DM) and total essential amino acids (TEA = 7.76 g/100 g
DM) among the study species. Porate pollen from male
flowers of the same species, however, had the lowest values.
The TAA is significantly higher in porate pollen from the
two functionally dioecious species (12.35 g/100 g DM for

### Table 1

| Amino acids | Hydrolyzed amino acid composition (mg/g) (±SE) |
|-------------|-----------------------------------------------|
|             | S. sejunctum M | F |
|             | S. ossicruentum M | F |
|             | S. ultraspinosum M | B |
| Essential   |                 |     |     |     |
| Histidine   | 3.991 (±0.031)  | 2.673 (±0.012) | 4.597 (±0.139) | 4.115 (±0.009) | 2.948 (±0.023) | 5.35 (±0.102) |
| Arginine    | 8.63 (±0.117)   | 8.021 (±0.016) | 10.143 (±0.374) | 9.245 (±0.013) | 6.964 (±0.444) | 12.039 (±0.254) |
| Threonine   | 6.611 (±0.110)  | 6.453 (±0.036) | 8.002 (±0.366) | 7.678 (±0.020) | 5.425 (±0.050) | 9.305 (±0.205) |
| Lysine      | 7.876 (±0.320)  | 8.147 (±0.089) | 11.565 (±0.802) | 11.554 (±0.178) | 8.980 (±0.094) | 12.835 (±0.423) |
| Methionine  | 2.401 (±0.028)  | 2.157 (±0.001) | 2.987 (±0.110) | 3.084 (±0.002) | 0.738 (±0.002) | 1.119 (±0.023) |
| Valine      | 8.185 (±0.153)  | 7.677 (±0.038) | 10.492 (±0.512) | 9.913 (±0.020) | 7.366 (±0.059) | 11.833 (±0.260) |
| Isoleucine  | 6.462 (±0.118)  | 6.042 (±0.028) | 8.336 (±0.402) | 7.936 (±0.041) | 5.839 (±0.035) | 9.363 (±0.200) |
| Leucine     | 10.758 (±0.200) | 10.316 (±0.069) | 13.853 (±0.677) | 13.243 (±0.046) | 9.591 (±0.064) | 15.711 (±0.346) |
| Non-essential amino acids |                 |     |     |     |
| Serine      | 7.612 (±0.117)  | 7.616 (±0.038) | 9.118 (±0.407) | 8.509 (±0.016) | 6.037 (±0.051) | 10.484 (±0.226) |
| Glycine     | 7.766 (±0.084)  | 6.944 (±0.003) | 8.996 (±0.320) | 8.200 (±0.012) | 5.980 (±0.047) | 10.536 (±0.193) |
| Aspartic acid | 13.835 (±0.500) | 14.253 (±0.164) | 18.465 (±1.148) | 18.12 (±0.202) | 12.520 (±0.303) | 19.885 (±0.715) |
| Glutamate   | 13.653 (±0.450) | 13.968 (±0.170) | 18.287 (±1.108) | 16.632 (±0.167) | 12.470 (±0.256) | 18.823 (±0.649) |
| Alanine     | 6.991 (±0.188)  | 6.883 (±0.045) | 9.110 (±0.519) | 9.083 (±0.083) | 6.387 (±0.083) | 10.102 (±0.277) |
| Proline     | 12.313 (±0.237) | 11.963 (±0.081) | 14.833 (±0.666) | 12.794 (±0.042) | 9.473 (±0.050) | 15.751 (±0.320) |
| Cysteine    | 0.306 (±0.002)  | 0.328 (±0.002) | 0.312 (±0.009) | 0.436 (±0.002) | 0.005 (±0.003) | 0.290 (±0.004) |
| Tyrosine    | 6.150 (±0.026)  | 5.428 (±0.023) | 7.170 (±0.199) | 6.517 (±0.012) | 4.573 (±0.031) | 8.347 (±0.014) |
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Differential Reward in "Male" Versus "Female" Solanum Pollen

The essential amino acid index (EAAI) is the geometric mean of the ratio of all essential amino acids (EAA) in the evaluated protein source relative to their content in a highly nutritive reference protein (Oser, 1959). In this case, the EAAI estimates the biological value of the pollen grains as food proteins for bees. The closer the EAAI is to 100%, the more nutritionally complete the substance is as a protein source (Oser, 1959). The EAAI ranged from 91.92% for inaperturate pollen from *S. ossicruentum* to 81.94% for porate pollen from bisexual flowers of *S. ultraspinosum*. The EAAI for both pollen from male and bisexual flowers of *S. ultraspinosum* was lower than the EAAI for either pollen from the functionally dioecious species (82.14% and 81.94%, respectively) (Table 3).

Because of the relative lack of data on other bee species, the minimum requirements of essential amino acids in the diet of honeybees (de Groot, 1953) are often used as a standard of measure for pollen nutritional quality. Analysis of percentage by mass for each amino acid allows comparison of Australian *Solanum* pollen to the established dietary needs of honeybees. Both porate and inaperturate

| Amino acids | Free amino acid composition (ng/100 g DM) (±SE) |
|-------------|-----------------------------------------------|
| **S. sejunctum** | **S. ossicruentum** | **S. ultraspinosum** |
| M | F | M | F | M | B |

**Essential amino acids**

| Amino acids | M | F | M | F | M | B |
|-------------|---|---|---|---|---|---|
| Histidine | — | — | — | — | 0.155 (±0.002) | — | 0.044 (±0.022) |
| Arginine | — | — | — | — | — | — | — |
| Threonine | 0.081 (±0.001) | 0.017 (±0.009) | 0.099 (±0.004) | — | — | 0.041 (±0.001) | 0.117 (±0.004) |
| Lysine | 0.119 (±0.001) | 0.051 (±0.004) | 0.167 (±0.001) | 0.122 (±0.001) | 0.094 (±0.003) | 0.270 (±0.015) |
| Methionine | — | — | 0.041 (±0.001) | — | — | — | 0.055 (±0.002) |
| Valine | 0.082 (±0.001) | 0.031 (±0.001) | 0.091 (±0.001) | 0.038 (±0.001) | 0.058 (±0.001) | 0.161 (±0.006) |
| Isoleucine | 0.174 (±0.020) | 0.259 (±0.067) | 0.288 (±0.024) | 0.277 (±0.060) | 0.238 (±0.008) | 0.309 (±0.005) |
| Leucine | 0.128 (±0.012) | 0.119 (±0.014) | 0.196 (±0.004) | 0.125 (±0.008) | 0.164 (±0.007) | 0.275 (±0.004) |
| Tryptophan | 0.184 (±0.008) | 0.243 (±0.020) | 0.232 (±0.005) | 0.261 (±0.016) | 0.248 (±0.006) | 0.292 (±0.004) |

**Non-essential amino acids**

| Amino acids | M | F | M | F | M | B |
|-------------|---|---|---|---|---|---|
| Asparagine | 1.068 (±0.005) | 0.146 (±0.008) | 1.427 (±0.001) | 0.406 (±0.002) | 0.084 (±0.002) | 0.448 (±0.016) |
| Serine | 0.819 (± 0.005) | 0.292 (±0.018) | 1.299 (±0.001) | 1.523 (±0.004) | 0.249 (±0.007) | 0.841 (±0.028) |
| Glycine | 0.194 (±0.002) | 0.051 (±0.003) | 0.193 (±0.001) | 0.050 (±0.001) | 0.060 (±0.001) | 0.167 (±0.003) |
| Aspartic acid | 0.244 (±0.001) | 0.298 (±0.028) | 0.478 (±0.002) | 0.055 (±0.001) | 0.204 (±0.003) | 0.254 (±0.014) |
| Glutamate | 0.488 (±0.001) | 0.030 (±0.015) | 0.649 (±0.003) | — | 0.244 (±0.006) | 0.160 (±0.007) |
| Alanine | 0.293 (±0.002) | 0.168 (±0.011) | 0.394 (±0.010) | 0.099 (±0.001) | 0.141 (±0.002) | 0.312 (±0.012) |
| Proline | 4.063 (±0.027)* | 1.362 (±0.072) | 5.460 (±0.008)* | 1.367 (±0.006) | 1.575 (±0.035) | 4.689 (±0.132)* |
| Cysteine | — | — | — | — | — | — |
| Tyrosine | 0.037 (±0.001) | — | 0.055 (±0.001) | — | — | 0.093 (±0.001) |
| Glutamine | — | — | — | — | — | — |

**Modified amino acids**

| Amino acids | M | F | M | F | M | B |
|-------------|---|---|---|---|---|---|
| Norleucine | 1.071 (±0.015) | 0.670 (±0.010) | 1.312 (±0.003) | 1.869 (±0.018) | 0.672 (±0.015) | 1.750 (±0.040) |
| Norvaline | 0.068 (±0.002) | — | 0.075 (±0.002) | 0.058 (±0.003) | — | 0.085 (±0.002) |
| GABA | 0.073 (±0.001) | 0.141 (±0.011) | 0.166 (±0.007) | 0.097 (±0.002) | 0.280 (±0.005) | 0.789 (±0.040) |
| Taurine | 0.068 (±0.001) | — | 0.111 (±0.001) | 0.110 (±0.002) | — | — |
TABLE 3 Total amino acid (TAA) content, total essential amino acid (TEAA) content, and essential amino acid index (EAAI) for pollen from “male” (M) flowers (porate pollen), functionally “female” (F) flowers (inaperturate pollen), and bisexual (B) flowers (porate pollen) of two functionally dioecious (S. sejunctum and S. ossicruentum) and one andromonoecious (S. ultraspinosum) Australian Solanum species (n = 3)

| Variable          | S. sejunctum | S. ossicruentum | S. ultraspinosum |
|-------------------|--------------|-----------------|-----------------|
|                   | M   | F  | M  | F  | M  | B  |     |     |     |     |     |     |     |     |
| TAA (g/100 g DM)  | 12.35| 11.89| 15.63| 14.71| 10.53| 17.18|     |     |     |     |     |     |     |
| TEA (g/100 g DM)  | 5.49 | 5.15 | 7.00 | 6.68 | 4.79 | 7.76 |     |     |     |     |     |     |     |
| EAAI (%)          | 90.85 | 86.03 | 90.54 | 91.92 | 82.14 | 81.94 |     |     |     |     |     |     |     |

**DISCUSSION**

Our findings provide evidence that pollen foragers on functionally dioecious Australian Solanum species receive different levels of nutritional reward depending on whether they forage on staminate individuals or functionally female individuals. In contrast to Buchmann’s (1986) findings of equivalent nutritive value (using nitrogen content as a proxy) in the pollen morphs of the functionally dioecious species S. appendiculatum, we found that staminate flowers produce porate pollen with higher levels of proteins and amino acids than do functionally female flowers in the functionally dioecious taxa used for this study (S. ossicruentum and S. sejunctum) (Table 5). Among our three study species, the highest levels of both proteins and amino acids were found in bisexual and staminate flowers, respectively, of the single andromonoecious taxon (S. ultraspinosum) (Table 5). Buchmann’s analysis utilized the macro-Kjeldahl method for protein estimation based on total nitrogen content, a method that Vanderplanck et al. (2014) found to generate an overestimate of protein concentration by three to eight times the actual value. Percentage nitrogen estimates using macro-Kjeldahl are more similar to total amino acid values, but still tend to be higher by a significant margin (Vanderplanck et al., 2014). While Buchmann found no significant difference between the two pollen morphs in S. appendiculatum utilizing the macro-Kjeldahl method, there was a lower mean nitrogen content (and thus protein estimate) in the inaperturate pollen—which mirrors our findings. It would be intriguing to see whether utilizing the more refined method established by Vanderplanck et al. (2014) would yield a statistical difference between the pollen morphs of S. appendiculatum.

The obvious nutritive differences between the pollen produced by the two morphologically bisexual flower types we studied, namely, in the functionally dioecious species and the andromonoecious species, could come down to one simple requirement: the production of a pollen tube. Inaperturate pollen grains (those produced by morphologically bisexual flowers of functionally dioecious species) are nonfunctional in the sexual sense because they do not have pores through which pollen tubes might germinate and

TABLE 4 Comparison of amino acid content in relation to minimum honeybee dietary requirements (a widely used standard) for pollen from “male” (M) flowers (porate pollen), functionally “female” (F) flowers (inaperturate pollen), and bisexual (B) flowers (porate pollen) of two functionally dioecious (S. sejunctum and S. ossicruentum) and one andromonoecious (S. ultraspinosum) Australian Solanum species

| Amino acid   | S. sejunctum | S. ossicruentum | S. ultraspinosum | Honey bee requirements* |
|--------------|--------------|-----------------|-----------------|-------------------------|
|              | M  | F  | M  | F  | M  | B  |                 |                          |
| Histidine    | 3.23| 2.25| 2.94| 2.8 | 3.11| 2.8 | 3.2              |                          |
| Arginine     | 6.99| 6.75| 6.49| 6.29| 7.01| 6.61| 4.5              |                          |
| Threonine    | 5.35| 5.43| 5.12| 5.22| 5.42| 5.15| 3.3              |                          |
| Lysine       | 6.37| 6.85| 7.4 | 7.86| 7.47| 8.53| 5.6              |                          |
| Methionine   | 1.94| 1.81| 1.91| 2.1 | 0.65| 0.7 | 1.4              |                          |
| Valine       | 6.63| 6.46| 6.71| 6.74| 6.89| 7   | 3.3              |                          |
| Isoleucine   | 5.23| 5.08| 5.33| 5.4 | 5.45| 5.55| 2.7              |                          |
| Leucine      | 8.71| 8.68| 8.87| 9.01| 9.15| 9.11| 5                |                          |
| Tryptophan   | 1.99| 6.26| 1.82| 3.95| 2.62| 5.69| 1.6              |                          |

*Note: Reported by de Groot (1953).
TABLE 5 Summary of differences in protein and amino acid content for pollen from “male” (M) flowers (porate pollen), functionally “female” (F) flowers (inaperturate pollen), and bisexual (B) flowers (porate pollen) of two functionally dioecious (S. sejunctum and S. ossicruentum) and one andromonoecious (S. ultraspinosum) Australian Solanum species. In each comparison, a plus sign indicates the greater value, the minus the lesser value.

| Comparison      | S. sejunctum | S. ossicruentum | S. ultraspinosum |
|-----------------|--------------|-----------------|-----------------|
| Total protein   | M            | +               | M               |
|                 | F            | −               | B               |
| Hydrolyzed amino acids | M | +               | M               | −               |
|                 | F            | −               | B               | +               |
| Free amino acids | M            | +               | M               | −               |
|                 | F            | −               | B               | +               |

deliver sperm cells. Even when inaperturate Solanum pollen grains reach a receptive stigma, pollen tube germination and fertilization do not occur and fruits are not set (Levine and Anderson, 1986; Anderson and Symon, 1989; Hayes, 2018).

Fascinatingly, we found that proline, the primary free amino acid required for pollen tube growth (Funck et al., 2012; Mattioli et al., 2012, 2018), is far less abundant in the inaperturate pollen of functionally female flowers in dioecious species. This finding makes some sense given that pollen tubes are unable to emerge from the inaperturate pollen grains produced by these flowers (Anderson and Symon, 1989; Hayes, 2018). Greater levels of total protein/amino acids in porate pollen of bisexual flowers of the andromonoecious species, where the pollen is equally germinable to that of unisexualy staminate flowers (Anderson and Symon, 1989; Hayes, 2018), may thus be due to higher amounts of proline and the related capacity for full development of pollen tubes from those grains. However, there is no proportional difference in proline content in the hydrolyzed amino acids, indicating that the difference in proline levels is due solely to free (most readily available for bee digestion) proline and not proline that is incorporated into proteins.

In functionally dioecious Solanum taxa, higher levels of reward presented by staminate flowers relative to functional females may reflect resource allocation to “male” function—the only significant reproductive investment given the lack of functional gynoecia in these flowers (which are present but vestigial). Functionally female flowers, meanwhile, may not be able to commit resources to pollen in the same way because of a downstream need to support fruit and seed development as per Bateman’s principle (Bateman, 1948; Wilson et al. 1994). While this makes sense in light of resource allocation (see Charlesworth and Morgan, 1991), it runs somewhat counter to theoretical notions (e.g., Martine and Anderson, 2007) connecting obligate outcrossing in the dioecious sexual system with directional flow of pollen from staminate plants to carpellate plants. Based on 15 years of unpublished observations (C. T. Martine) on sex ratios in northern Australian Solanum taxa, wild, functionally dioecious populations are nearly always ca. 1:1 male to functional female; the same has been observed for seedling recruitment in ex situ plant culture from seed (C. T. Martine, unpublished data). When one couples this understanding of sex ratios with observed constraints on flower production—that is, while female flowers are borne solitarily, males are in inflorescences of anywhere from ~10–80 depending on the species (Symon, 1981), we can assume that in any given population, male flowers with porate pollen of higher nutritive quality far outnumber female flowers with inaperturate pollen of lesser quality. How then is reproductive fitness maintained when one might imagine little reason for a pollinator to cease foraging on a given male plant to move to a lesser-resourced female plant (rather than another male), as required by the dioecious condition? Three factors may come to bear:

1. In all Australian functionally dioecious Solanum species, the corollas of functionally female flowers are larger than those of males (see Symon, 1981; Anderson and Symon, 1989; Brennan et al., 2006; Barrett, 2013; Barrett and Hough, 2013; Martine et al., 2013, 2016) and may thus elicit visitation (Galen, 1989; Cresswell and Galen, 1991; Duffield et al., 1993), although a shift to a female plant would have to override (or follow) potential preferences for the larger inflorescence displays of male plants. To date, only one study (Anderson and Symon, 1988) has examined pollinator visitation patterns in our lineage of focus, finding “no obvious difference in behavior” of insects visiting male versus functionally female flowers in five functionally dioecious taxa.

2. In general, pollen from buzz-pollinated flowers has been found to be higher in nitrogen, protein, amino acids, and caloric content than that of closely related bee-pollinated, “non-buzz” species (Buchmann, 1983, 1986). Differences within a given Solanum species may thus matter little relative to the less-nutritive options offered by “non-buzz” sympatric species.

3. Although the amount of pollen produced in each floral morph may differ slightly (Anderson and Symon, 1989), foraging adult bees might interpret them as roughly equivalent (and thus not modify foraging behavior at the
level of the individual flower). Differences in the nutritive value of those loads might only manifest where the pollen is primarily consumed (i.e., the larvae) and any slight disadvantage to consuming inaperturate “female pollen” is swamped out by the greater overall presence of porate “male pollen.” More work is needed to understand the effects of differential pollen quality on larval development, as well as whether or not adult bees are capable of discerning the nutritive differences we have found.

Pollen quality, as measured in protein and amino acid content, was higher in bisexual flowers of the andromonoecious S. ultraspinosum than in either of the functionally dioecious taxa. In fact, the porate pollen produced by the morphologically and functionally bisexual flowers of S. ultraspinosum showed the highest levels of potential nutriment—although the protein and amino acid levels in bisexual flowers far outpaced those in males (Tables 3, 5). While we only studied a single andromonoecious taxon (of ~14 in the Australian Monsoonal Tropics, as per Lacey et al., 2017; McDonnell et al., 2019), the key to understanding high pollen quality in andromonoecious flowers may be related to evolutionary transitions currently under study (Martine et al., 2019; McDonnell and Martine, 2020; A. J. McDonnell and C. T. Martine, unpublished manuscript). Forthcoming results as well as observations by Bean (2012 onward) suggest that the andromonoecious system in Australian Solanum may be fairly labile, varying in strength of expression (that is, number of stamine flowers relative to bisexual flowers), co-occurring in the same clades with fully hermaphrodite taxa, and (perhaps at least once) transitioning to functional dioecy (Martine et al., 2006, 2009, 2019; McDonnell and Martine, 2020; A. J. McDonnell and C. T. Martine, unpublished manuscript). Within this lineage, the relative lability of sexual systems and selective pressures related to outcrossing and maintaining fidelity of pollen-focused pollinators have each likely influenced whether and how shifts in pollen nutritional quality have occurred. In andromonoecious taxa where stamine flowers and bisexual flowers share the same inflorescence, the greatest chance of outcrossing may lie with attracting bees to visit bisexual flowers as they arrive to a plant (as per Martine and Anderson, 2007)—both by having larger corollas and greater pollen quality than nearby stamine flowers. Conclusions about pollen resource allocation within andromonoecious Solanum in Australia will be clarified by further studies involving more taxa and incorporating current knowledge of this sexual system in the genus (e.g., Diggle, 1991, 1994; Diggle and Miller, 2003; Miller and Diggle, 2004; Quesada-Aguilar et al., 2008). In the meantime, we here report the first nutritional data of its kind for an andromonoecious Solanum taxon as a comparison to closely related dioecious congeners and a starting point for future studies.

In summary, lower levels of total protein and free amino acids in the functionally female flowers of functionally dioecious Australian Solanum species may translate into lower nutritive potential of pollen for foragers who visit them. The apparent ramifications of foraging on functionally female flowers may be rendered moot, however, by the significantly greater occurrence of male flowers and the lower likelihood of inaperturate pollen being collected and consumed. Still, many questions remain regarding how the differences in pollen nutrition identified in this study might affect foraging patterns and larval development of bees, rates of outcrossing, and, fundamentally, the evolution and maintenance of the functionally dioecious sexual system in Solanum.

ACKNOWLEDGMENTS
The authors thank Mark Spiro, Mitch Chernin, Charles Clapp, and Monica Hoover for guidance provided to J.R.N. on aspects of the thesis from which this manuscript was developed; Jason Cantley for assistance with initial study design; Ariel Antoine for help with pollen collection; and Beth Capaldi for valuable discussion of bee-foraging behavior. Funding: David Burpee Endowment at Bucknell and Botanical Society of America Undergraduate Student Research Award. The manuscript was improved significantly thanks to comments from AJB reviewers and editors.

AUTHOR CONTRIBUTIONS
J.R.N. performed all experimental research and wrote the initial draft; J.H., A.J.M., and C.T.M. developed the project idea; A.J.M. managed all plant-based work; J.H. and C.T.M. led the final manuscript writing.

DATA AVAILABILITY STATEMENT
Raw data files can be accessed via FigShare: https://figshare.com/s/724cb76c87c2968c0f20, https://figshare.com/s/375086ad8bca1353b2f.

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