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POLLINATION ECOLOGY AND FLORAL FUNCTION OF BROWN'S PEONY (*PAEONIA BROWNII*) IN THE BLUE MOUNTAINS OF NORTHEASTERN OREGON

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Abstract— Brown's peony, *Paeonia brownii* (Paeoniaceae), is one of only two peony species native to the Western Hemisphere, yet its pollination ecology and breeding system have never been documented. Using flowering individuals of an endemic colony in the Blue Mountains of Oregon, U.S., we investigated the peony's pollination system and floral function. We also examined pollen/carpel interactions through experimental pollinations aided by fluorescence microscopy. *Paeonia brownii* appears to be self compatible and mostly protogynous with floral traits of a generalist pollination system. The flowers appear to attract insects by producing abundant floral nectar secreted from lobes of a perigynous disc throughout their 9-15-days of anthesis. The most common pollen vectors were wasp queens (Vespidae), the large flower fly *Criorhina caudata* (Syrphidae), and females of *Lasioglossum* spp. (Halictidae), all of which foraged exclusively for nectar. Whether collected from foraging wasps and flies, anthers, or stigmas, about half the pollen grains appeared fertile. The number of ovules per carpel was about 19. Seed set (seeds/ovule) of naturally pollinated flowers was about 20% with about 4 viable seeds per follicle. The number of fertile pollen grains transferred to the stigma under natural conditions was highly variable but generally low, which may have contributed in part to the low rate of seed set. This study raises further questions about the role of pollen sterility, floral nectar and vespid wasps in shaping a pollinator system that is unusual in *Paeonia.*

Keywords: Breeding system, Floral nectar, Paeonia brownii, Pollen, Pollination

INTRODUCTION

Despite a long history of breeding and propagation that continues today, most wild populations of Eurasian species of the genus *Paeonia* are listed as threatened or under protection. Because of their enduring popularity as ornamental and medicinal plants, continual wild collection and habitat loss threaten the existence of endemic populations (Page 2005). Concerns for the conservation of these Paeonia species' remnant populations are prompting research that leads to a greater understanding of their reproductive biology including studies on pollination and breeding systems (Luo et al. 1998; Jing & Zheng 1999; Zhou et al. 1999; Sánchez-Lafuente et al. 1999; Sánchez-Lafuente 2002; Andrieu et al. 2007).

Paeonia brownii Douglas ex Hook. and *P. californica* Nutt., the two species that comprise section Onaepia, are endemic to Pacific western North America. Unlike the Eurasian peonies, they have not been cultivated and are almost always found in the wild. Schlising (1976) determined that protogyny, floral presentation and abundant pollen in flowers of *P. californica* were indicative of allogamy and described a generalist pollination system that incorporated the naturalised honeybee, *Apis mellifera*. *Paeonia brownii* shares similar morphology and floral traits with *P. californica* (Stern 1946); however, *P. brownii* is endemic to locales at higher latitudes and elevations and the hardier of the two species (Stebbins 1938). Stebbins and Ellerton (1939) found and described cytological differences between *P. brownii* and *P. californica*. These differences between the two species may have evolved through allopatry and endemism to different habitats (Stebbins & Major 1965) while remaining under "phylogenetic constraint" (sensu Baker & Baker 1983).

Cytological studies of *P. california* and *P. brownii* revealed chromosomal irregularities during meiosis, including translocation heterozygosity, which can result in gametophytic sterility (Stebbins & Ellerton 1939; Walters 1962; Zhang and Sang 1998). Pollen viability of *P. brownii* and *P. californica* had been ascertained to be as low as 20% and 10%, respectively (Stebbins & Ellerton 1939; Walters 1956; Zhang & Sang 1998). However, Walters (1962) suggested that the high number of anthers more than compensated for pollen sterility and that ovule fertility is not the limiting factor in seed set as evidenced by high seed production.

Grant (1975) proposed that *P. californica* was probably a self-pollinating species; however, Schlising (1976) noted a higher seed set in experimental cross- than self-pollinated flowers and a high frequency of anthophilous insects. If *P.*

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brownii is primarily cross-pollinated, pollen sterility may well be a factor in the quantity and quality of the vector pollen load and the amount of fertile pollen deposited on the receptive stigma (Inouye et al. 1994). However, a first investigation should be to identify the potential pollinator system that is associated with this species' endemism and consider its effectiveness.

The purpose of this study is to investigate floral functions, pollen viability (fertility), and breeding and pollination of *P. brownii* flowers in relation to their potential to influence fecundity. A major objective is to ascertain if this species is self-pollinating or displays active self-incompatibility as has been demonstrated in other species (Zhou et al. 1999). A specific objective is to test the null hypothesis that there are no differences in the pollination system between *P. brownii* and other *Paeonia* species. Our intent is that by characterising floral traits and components of the pollination system unique to *P. brownii*, we will contribute to a broader conceptual model of the pollination systems in the genus *Paeonia*.

MATERIALS AND METHODS

Study species

Paeonia brownii Dougl. ex Hook. (Paeoniaceae) is a long-lived perennial herb (Saunders & Stebbins 1938; Page 2005) that occurs primarily in the semi-arid regions of far-western North America. A relict of mesic forests once widespread in the northern hemisphere during the Paleocene-Pliocene period, it is patchily distributed from southern British Columbia to the Sierra Nevada in California and east to Wyoming and Utah where it is rare (Hitchcock et al. 1964; Stebbins & Major 1965). Similar to other *Paeonia* endemics, *P. brownii* is adapted to habitat conditions that consist primarily of cold winters and warm-hot summers (USDA 1988; Page 2005). It commonly occurs in pine forests and sagebrush steppe, but is also found in cooler fir and aspen communities at higher elevations up to about 2500 m. Individuals are widely scattered and rarely form dense colonies but over time can become large plants. Shoots first appear in early spring often through snow. It flowers from April-June depending upon elevation.

At maturity *P. brownii* is multi-stemmed, growing from a large, fleshy root. Each stem is semi-decumbent and has 5-7 biternate, bluish green, glabrous and somewhat thick leaves with deeply dissected leaflets having purple-tinged edges in cold temperatures (Hitchcock et al. 1964). Flowers are large, nodding, solitary and terminal on a branching stem. The 5-6 sepals are a purplish green, ovate to sub-orbicular, overlapping and cupped; the 5-10 orbicular petals grade in colour from brownish-maroon to yellow on the margins. The polyandrous flower has yellow stamens and a perigynous, fleshy nectariferous disc with greenish-yellow lobes 2.5-3.0 mm high. The large gynoecium consists of 2-6 glabrous yellow-green to yellow-red carpels, each having a short style surmounted by a curved stigma appearing as a narrow ridge at the apex of the style. Fertilised carpels mature into follicles that become leathery at time of dehiscence (Fig. 1). Seeds are yellowish-tan to black, round-ovoid and 6-11 mm in diameter. *Paeonia brownii* is a diploid (2N = 10) species (Stern 1946; Hitchcock et al. 1964).

Study sites

The study was conducted from April-May, 2003-2005 on flowering plants selected from a colony of *P. brownii* composed of 100-200 individuals distributed over 4 ha (45° 32.622' N, 117° 58.680' W) on the property of the Grande Ronde Overlook Wildflower Institute Serving Ecological Restoration. This study area is located within the 2.4 million ha Blue Mountains Ecological Province in northeastern Oregon, U.S. A different colony was located 320 km west of the Blue Mountains study area on the eastern flank of the Oregon Cascades Range at about 1000 m elevation. It was visited to sample nectar from flowering individuals for chromatographic analysis and for additional observation of floral foragers.

The Blue Mountains site consists of an upland prairie adjacent to a coniferous forest located on basalt-substrate, scabland of a shield volcano 1036-1076 m in elevation. The plant community is composed of a mixture of grasses, broadleaf herbs, and shrubs endemic to bunch-grass prairies and open pine forests of the Blue Mountains. Other putatively entomophilous species associated with *P. brownii* include *Aconitum columbianum, Balsamorrhiza hookeri, B. sagitatta, Ceanothus sanguineus, Clarkia pulchella, Clematis hirsutissima, Delphinium* spp., *Eriogonum heracleoides, Lomatium dissectum, L. triternatum, Lupinus sulphureus, Penstemon* spp., *Phacelia heterophylla, Potentilla glandulosa, P. gracilis, Ranunculus glaberrimus, Rosa gymnocarpus, R. nootkana, Symphoricarpos albus, Vicia americana* and *Wyethia amplexicaulus*.

Floral presentation and reproductive traits

For two weeks beginning 09 May 2004, we randomly selected a single bud from each of 16 randomly selected plants and observed daily developmental changes in floral structure using 10X optical glass magnifiers (Donegan Optical Co.). We recorded dates when the following developments appeared in each flower: corolla opening, nectar secretion onset, stigmatic receptivity onset (the fold of the stigmatic crest are open, pale, moist, and papillose) and ending (stigmatic crest folds together, darkens, becomes crustose with senescent papillae), anther dehiscence, and petal abscission. We collected an open flower (male phase) from each of 33 randomly selected plants in late April and May 2004. Fixation and storage of the flowers followed Goldblatt et al. (2004). Under a dissecting microscope we counted the number of lobes of each flower's floral disc. Anthers were counted using enlarged digital images of a subset of 8 undamaged flowers in mid-late anthesis when all anthers were exposed.

In early May 2004 we collected a single flower that completed flowering (petals and anthers senesced and dropped) from each of 42 randomly selected plants. Under the microscope we counted the number of ovules per ovary of a carpel removed from each flower's gynoecium. In mid-May we also counted the number of carpels, and developing

follicles taken from each of 18 randomly selected flowers that finished flowering. In mid June, 24 follicles were collected by random selection and seeds were counted and evaluated. Empty seeds appeared shrivelled or poorly developed; when cut, endosperm was lacking or much reduced and necrotic compared to that of developed seeds. Seeds that appeared viable were smooth, yellow to tan-brown, medium-to-large and filled with creamy white endosperm with a rudimentary embryo visible at the basal end.

Observation and collection of floral foragers

Observation of insects visiting the flowers of *P. brownii* in May 2003 and in April-May, 2004-2005 constituted about 52 hours. We noted which insects visited flowers beginning on the first day of anthesis, which was defined as beginning when petals parted enough to expose the tips of the carpels. We recorded whether insect visitors foraged for nectar and/or pollen and whether they could be found on the flower throughout the floral lifespan. We used binocular magnifiers to determine whether insects contacted the stigmas while they visited the flower.

We took pollen load data on 100 insects over three seasons using the following procedures: Individual insects were caught with a butterfly net after they were observed contacting floral organs. After they were killed in a jar containing ethyl acetate, pollen grains were washed from the insect specimens with ethanol, pinned and labelled following the protocol of Goldblatt & Bernhardt (1990). Pollen grains from the insects were mounted on a glass slide, stained with Calberla's fluid (Ogden et al. 1974), identified and counted under a compound microscope. As different insects were killed in the same jar, even though few grains were dislodged from the insects, pollen of a plant species was listed as present only after we counted ≥ 20 grains of the same pollen morphotype on the slide. Vouchers of insect specimens were identified and deposited in collections by entomologists at the American Museum of Natural History (New York; J. Carpenter, wasps and J. Rozen, bees), Smithsonian Institution (Washington, DC; C. Thomson, flies), Snow Entomological Museum (Kansas State University; C.D. Michener, bees) and the Museum of Southwestern Biology (University of New Mexico; L. Larkin, bees in the genus *Andrena*).

We also collected caterpillars that were found inside flowers. Some specimens were preserved in 70% ethanol. Other live specimens were brought back to Saint Louis University and reared in insect cages in the laboratory of J.C. Fortier. Moths emerging from pupation were killed and pinned. Pinned moths, along with caterpillars and pupal cases, were sent for identification to M. Pogue, Smithsonian Institution, Washington DC.

Pollen analysis

We removed pollen grains of *P. brownii* from sampled insect visitors, anthers or stigmas and stained them with Calberla's solution, a fuchsin stain that allows the pollen structures to be assessed under the microscope. We classified the grains as appearing either potentially fertile or sterile using the following criteria: pollen walls of fertile grains stained pink as they hydrated or began to form pollen tubes (for grains on stigma); all three exine apertures including gray, granular cytoplasm were visible under light microscopy. In contrast, the walls of sterile grains stained a deep reddish-purple; never hydrated or germinated; one or all exine apertures were not discernible because of the collapsed wall folding up on itself with no visible cytoplasm inside the grain.

To determine whether sterile pollen grains were being transported by insect visitors, we counted the first 50 visible grains on a pollen wash slide of an insect and classified them as either sterile or fertile using the above protocol. Pollen samples were taken from 5 vespid wasps and 9 syrphid flies (*Criorhina caudata*) only because these insects were observed to contact receptive stigmas while foraging for nectar but were never observed ingesting pollen or actively foraging on dehiscent anthers for pollen (sensu Bernhardt 1996). To compare fertile versus sterile grains of insect-born pollen with non-transported grains remaining inside anthers, we took a pollen smear from a dehiscent anther of a flower collected one per day from each of 15 randomly selected plants over a two-week period in May 2004. Pollen grains of each anther were stained and counted as described above. To determine the proportion of sterile pollen that was deposited onto receptive stigmas under natural conditions, from the same flowers we selected 10 visibly receptive stigmas that had a minimum of 10 visible pollen grains. We counted and classified all the grains of *P. brownii* pollen detected on each stigma using the protocol for classification described above.

Pollen-carpel interactions

To determine natural rates of pollination over the flowering period, we collected a flower with a visibly receptive stigma (see above) from each of 5-6 plants on 7 separate dates after 1200 h, from 14 April through 03 May 2004. Flowers with petals expanded sufficiently to expose one or more stigmas were tagged and harvested 48 h later, when they were in the male phase of anthesis. Once picked, each whole flower was fixed and stored following Goldblatt et al. (2004). To view and count the number of pollen grains on the stigmas and the number of pollen tubes penetrating carpel tissue, one carpel from each flower was selected at random, excised and soaked in a 5% sodium sulfite solution in de-ionised water at 60 °C for one hour and 40 min. The carpel was removed from the softening solution and washed in room temperature with de-ionised water. Each carpel was placed on a separate glass slide. A razor blade was used to longitudinally bisect the organ, which caused the thick cuticle to rupture, and the cuticle was stripped off the epidermis with forceps prior to squashing. Final preparation of the carpel halves for spreading tissues, staining and viewing under fluorescence microscopy followed Goldblatt & Bernhardt (1990). We combined counts of pollen tube germination and penetration for each pair of carpel halves. Counts included the number of germinating grains on the stigma, the number of pollen tubes visible in the style, and the number of tubes in the ovary loculus (including those adjacent to and/or penetrating ovules).

To determine whether carpels showed any recognizable early- or late-acting, self-incompatibility responses, in 2004 we selected a single, mature flower bud on each of 33 plants during the flowering period in early May. Each labelled bud was randomly selected for one of four pollen enhancement treatments (Vance et al. 2004): 1) Autogamous pollination (AP). The bud was covered securely in a tulle bag 12-48 h before anthesis and remained isolated for the duration of the experiment. 2) Hand applied self-pollination (HSP). The bud was isolated as for AP until the day of anthesis when the bag was removed briefly and all stigmas in the flower were smeared with a coat of pollen (visible under a binocular magnifier) taken from dehiscent anthers of an open flower on the same plant (geitonogamous pollination). The bag was replaced for the duration of the experiment. 3) Hand applied cross-pollination (HCP). The procedure was the same as for (HSP) except the pollen was transferred from a different flower on a plant separated by a distance > 1m. 4) Natural insect- and/or self-pollination (IP). The mature bud was not bagged and the flowers were collected as described in text above.

All flowers hand-pollinated in this study were harvested and fixed three days after the petals opened when the stigmatic surface appeared necrotic or crustose but before stigmas shrivelled, making it impossible to count both the full number of pollen grains adhering to and germinating on the stigmatic surface and pollen tubes penetrating the transmission tissue (Bernhardt & Dafni 2000). Fixation, storage and preparation for fluorescence microscopy followed the protocol described above.

Nectar analysis

In 2005, a flower bud on each of 40 plants was bagged for floral nectar collection to determine volume and concentration un-confounded by nectar feeders and uncontaminated with insect saliva and debris. After petals opened, we collected nectar from 39 flowers. We withdrew nectar in the concavity at the base of each lobe of the floral nectary (disc) using a 10 µL capillary tube. The dissolved solute content volume and concentration of the extracted nectar were recorded with a Bellingham and Stanley refractometer (Eclipse model 45-03; 0-50%). Volume and concentration of dissolved solutes were co-referenced with flower phase. The following year we sampled nectar from flowering individuals at the eastside Cascades location for analysis of nectar sugars and amino acids. For quantitative analysis of nectar constituents, we drew nectar into 100 µL capillary tubes from the floral nectary in each of 11 flowers that were pre-bagged as buds. The nectar was spotted on filter paper, labelled and express mailed to the Biology and Agriculture Chromatography Laboratory at Brigham Young University, Provo, UT, U.S. Sugars were separated using gas chromatography (Hewlett Packard 5890 GC, Palo Alto, CA, U.S.) equipped with a flame ionization detector and identified by comparing retention times with standards. Amino acids were separated and analysed by HPLC (Agilent 1100 Series, Santa Clara, CA, U.S.) using fluorescence detection following the method of Cohen & Michaud (1993).

Statistical analysis

Data were statistically analysed using Statgraphics Plus statistical software version 5.1. (Manugistics, 2000, Rockville, MD, U.S.). We used one way ANOVA for analysis of pollen tube germination among treatments applied to characterise breeding system and the t-test for differences in pollen viability among different sources of pollen. Significant differences in means were tested at the 95% confidence level. Confidence intervals among the means were determined using Bonferroni intervals. Three statistics (Cochran's C, Hartley's and Levene's tests) were used to test for equal variance at $P \leq 0.05$. Where assumptions of equal variance were not met, values were log transformed to remove dependence of the standard deviation on the mean. Where assumptions were not met for normal distribution, the Kruskal-Wallis or Mann-Whitney nonparametric test was used to compare medians instead of means. Multiple regression analysis was used with the R^2-statistic to develop best-fit linear models that explained variation in factors associated with nectar and seeds. ANOVA was used to test for a statistically significant relationship between the variables at the 95% confidence level.

RESULTS

Floral traits and life span

In early April 2004 at the Blue Mountains site, *P. brownii* had multiple spreading stems (Fig. 1a). Each of the flowering stems (2-10 depending on the size of the plant), bore a single, large, globose terminal bud (Fig. 1a). Anthesis of flowers began in mid April 2004 and 2005, and continued through mid May. The mean floral lifespan of a flower was 11.4 d (n = 16) and ranged from 9-15 d. During the first several days of anthesis, the corolla expanded enough to expose each stigma on the carpel's axis but partially occlude the indehiscent anthers (Fig. 1b). The moist stigmatic ridge of each carpel opened and expanded until the receptive stigma exposed the turgid, unicellular papillae. The mean number of days of stigma receptivity was 2 (range 1-4) or 18.7 % of the floral lifespan. Over the floral lifespan, as carpels increased in size they became more prominent and their colour changed from yellowish green to yellowish red (Fig. 1c,d,e).

The mean number of anthers in the androecium was 84.4 ranging from 63-92 (n = 10). Each anther was born on a thick filament that was elongated at time of dehiscence. The mean number of days in the male or staminate phase was 9.8 or 85.9% of the floral lifespan. In succession centrifugally, 6-12 anthers dehisced each day (Fig. 1d). In 6 of 16 flowers observed daily over the floral lifespan, a few anthers closest to the carpels began to dehisce while surfaces of the stigmatic crests were still fully expanded. The length of overlapping male and female phases of those flowers was about 1 day or 5% of the floral lifespan. Over the course of the male phase while the petals remained attached to the flower, the stigma and short style shrivelled, and any remaining exposed papillae on the closed stigmatic crest turned brown and crusty (Fig. 1d). The fleshy, yellowish, lobed disc that encircled the carpels at the base of the

FIG. 1. (a) *Paeonia brownii* bearing terminal globose buds. (b) Opening bud exposing receptive stigma. (c) Early anthesis with visible carpels, anthers. (d) Flower mid-anthesis, anthers visibly dehiscent, nectariferous disc at base of carpels exposed. (e) Flower with petals and stamens removed to show prominent lobes of nectary (arrow). (f) Opened flower revealing moth larva and damage from herbivory. (g) Solitary bee attracted to nectar. (h) *Criorhina caudata* (Syrphidae), *Bombus* mimic and a primary pollinator of *P. brownii*. (i) *Dolichovespula arenaria* (Vespidae) queen on flower in staminate phase.

perianth formed an incomplete barrier between the androecium and gynoecium (Fig. 1d,e). When the floral phase was over and the lobes were no longer secreting, they also shrivelled and appeared faded (Fig. 1f).

Every disc sampled from flowers in the pistillate or staminate phase secreted nectar. The nectar was sweet with a bitter aftertaste, reminiscent of the floral odour which was weakly bitter and unpleasantly scented. The same odour became far more pronounced in leaf and stem vegetation when it was broken, bruised or damaged by predation (Fig. 1f).

Flower visitors

We identified insects representing 16 different taxa in the Orders of Diptera and Hymenoptera that were collected on open flowers of *P. brownii* from 2003-2005 (Tab. 1). Of 100 visitors recorded 80% were represented by members of 4 taxa: *Lasioglossum* spp. (Halictidae) (27%) (Fig. 1g), *Criorhina caudata* (Syrphidae) (17%) (Fig. 1h), *Dolichovespula arenaria* (Vespinae) (19%) (Fig. 1i) and *Polistes aurifer* (Polistinae) (17%). Six wasp species represented 45% of the visitors recorded.

Insects of other taxa were observed visiting *P. brownii* flowers at the eastside Cascades location (Vance pers. com., data not shown). Among them were large flies in families Tachinidae and Sarcophagidae, and a single *D. Adulterina* queen, an obligatory social parasite of *D. arenaria* (Greene et al. 1978; Buck et al. 2008). Unidentified small to medium bees visited the flowers at all phases of its life span. Ants (*Formica* spp.) were commonly observed crawling on flowers and buds (Vance 2012).

We observed vespid wasps from the last two weeks of April until the end of the flowering period in May. Although wasps were observed on flowers as early as 0930, their peak periods of activity occurred from 1430-1630 h during the warmest part of the day. All of the wasp species visited flowers at all phases of the floral life span. All were observed foraging exclusively for nectar. *Dolichovespula arenaria* tended to visit all open flowers on the same plant and/or flowers on individual plants with overlapping stems. Commonly, we observed as many as 3 wasps concurrently visiting the same flower. All wasp visitors were native except for *P. dominula,* which we observed building a nest near *P. brownii*. All wasps collected on *P. brownii* were fertile, egg-laying females ranging 13-20 mm in length (Tab. 1)

Female, solitary bees in the genus *Andrena* about 11 mm in length and *Lasioglossum* (*Evylaeus*) ranging from 6-7 mm were most often observed foraging for nectar in staminate-phase flowers. Those recorded were seldom seen contacting the elevated, receptive stigmas in early female phase flowers. Bee visits to female-phase flowers were observed a total of four times over two seasons at the Blue Mountains site. Other medium-large bees including *Apis mellifera* (12 mm), *Eucera* spp. (6-14 mm), and *Bombus mixtus* (16 mm) were infrequent visitors to *P. brownii* over the two seasons.

Of the dipterans collected, 19 were syrphid flies, 18 of which were *Criorhina caudata*. These *Bombus* mimics are mostly black, thickly pilose and large, ranging from 13 to 18 mm in length (Fig. 1 h). *Criorhina caudata* was active on *P. brownii* from April until the beginning of the first week in May in both years. These flies remained in May but appeared to forage preferentially on *Balsamorhiza* spp. and *Delphinium nuttallianum*. Throughout early April, on overcast days with temperatures ranging from 0 to 16 °C, male and female *C. caudata* were observed moving from flower to flower (< 1 min/flower) taking up nectar through their siphon-like probosces. Peak activity was approximately from 0900–1200 h. We observed flies contacting receptive stigmas as they moved from lobe to lobe of the nectary. They also attempted to probe mature buds in which the petals had not yet separated. All *C. caudata* that were analysed for pollen had > 200 pollen grains of *P. brownii* typically on their heads and thoraces.

Of 85 insect specimens that carried pollen from *P. brownii*, 41% also carried pollen from other plant species (Tab. 1). The pollen grains of co-flowering species most frequently found on insects were from *B. hookeri, B. sagittata,* and *D. nuttallianum*. Species in which the majority of the specimens carried only *P. brownii* pollen were the fly *C. caudata* and the wasps *D. arenaria* and *P. aurifer*, none of which foraged for pollen. Of the 37 wasps that carried pollen, 6 carried > 100 grains on their heads, legs and thoraces; 24% had grains of species other than *P. brownii*.

TABLE 1. Pollen load analyses of insects collected on flowers of *Paeonia brownii* in the Blue Mountains of northeastern Oregon, April-May 2003-2005.

Insect taxon	*P. brownii* only	*P. brownii* + other	Other only	No pollen
Diptera (Syrphidae)				
Criorhina caudate	12	5	0	0
Eristalis hirta	1	1	0	0
Subtotals	13	6	0	0
Hymenoptera (bees)				
Andrena astragali	1	2	0	0
Andrena lawrenci	1	0	0	0
Andrena nigrocaerulea	0	1	0	0
Apis mellifera	1	0	0	0
Bombus mixtus	0	1	0	0
Eucera actuosa	0	1	0	0
Eucera sp.	0	0	1	0
Lasioglossum spp.	6	16	2	3
Subtotals	9	21	3	3
Hymenoptera (wasps)				
Dolichovespula arenaria	10	5	0	4
Polistes aurifer	10	2	1	4
Polistes dominula	1	0	0	0
Vespula atropilosa	3	0	0	0
Vespula consobrina	2	1	0	0
Vespula pensylvanica	2	0	0	0
Subtotals	28	8	1	8
Grand totals	50	35	4	11

Of 19 flies that carried pollen, *C. caudata* generally carried more pollen than the wasps; the number of grains ranged from > 100 to > 200. About 32% of the *C. caudata* also carried pollen from other co-blooming species (Tab. 1). The number of grains that bees carried varied widely among bee species; grains carried by *Andrena* and *Lasioglossum* species ranged from 10 to >100; about 72 % of the bees carried pollen grains of species other than *P. brownii*.

Pollen analysis

Light and fluorescence microscopy showed that pollen grains from each of the sources (flies, wasps, anthers, stigmas) consisted of a mixture of sterile and fertile pollen. Fertile pollen from all sources combined was 51.8 ± 5% and highly variable ranging from 20 to 92% (Tab. 2). The difference between wasps and flies in the mean number of potentially fertile pollen grains they carried was significant ($t = 3.6$, $P < 0.004$) (Tab. 2). Otherwise, no significant differences were detected in the mean percentage of potentially viable pollen between each source carried by the insects or found on stigmas or anthers (Tab. 2).

Pollen-carpel interactions

The mean number of fertile pollen grains on the stigmatic surface of *P. brownii* flowers differed significantly with sampling date ($F = 3.58$, d.f.= 32, $P = 0.01$) (Fig. 2), and was highest on April 22, ranging from 21-157 (n = 5). The pollen tubes in the transmission tissue of the style were fewer in number than on the stigmas and their means did not differ significantly among the sampling dates ($F = 0.22$, $P = 0.96$). In the ovary, pollen tubes were only detected 48 h after anthesis on three dates in late April and one in early-May (Fig. 2). Zhou et al. (1999) reported that at least 48 hours were required for pollen tubes to penetrate the ovary of *P. jishanensis*. Pollen tube growth may have been slowed by the low night-time temperatures at the site (mean $t_L = 0.5$ °C). Minimum daily temperatures were never higher than 4 °C over the entire flowering period from mid April to mid May (Oregon Climate Service 2004 data, Elgin, Oregon Climate Station, Oregon State University).

TABLE 2. Mean percentage of potentially fertile pollen deposited on stigmas, and of 50 pollen grains collected from flies (Syrphidae), wasps (Vespidae) and anthers of *Paeonia brownii* and analysed under a compound microscope. Results of t-tests for significant differences between the means at $P < 0.05$ are shown by different letters; c.i. = 95% confidence intervals for means.

Pollen source	Fertile pollen (%)	(c.i.)	*n*
Fly	63.3[a]	(57.7, 68.9)	9
Wasp	43.2[b]	(25.9, 60.5)	5
Anther	50.3[ab]	(41.4, 59.2)	15
Stigma	58.5[ab]	(43.4, 73.6)	10

Significant differences were detected among the four pollen treatments used to evaluate pollen-pistil interactions ($F = 7.13$, $P = 0.002$). The hand pollinated stigmas (HCP and HSP) contained significantly more fertile pollen grains than did stigmas exposed to insect pollinators (IP) or bagged flowers that only allowed autogamy (AP) (Tab. 3). Four of the AP-treated stigmas completely lacked pollen grains. Also the number of pollen tubes counted in the styles differed significantly among the treatments (Tab. 3). In general, fewer pollen tubes were counted growing through the transmission tissue of the style than on the stigma, but there were significantly more pollen tubes in the styles and ovaries of the HSP- and HCP- treated carpels than the IP or AP treated carpels (Tab. 3, Fig. 3 c,d).

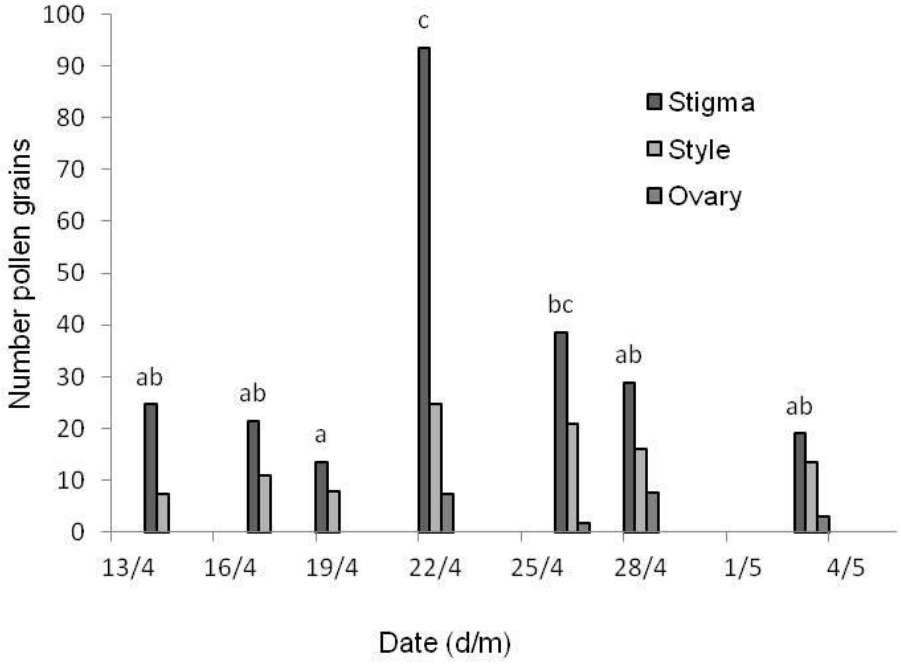

FIGURE 2. Mean number of visibly fertile and/or germinated pollen grains on stigmas, and pollen tubes in styles and ovaries of *Paeonia brownii* on various dates from 14 April − 3 May 2004. Different letters denote significant difference in mean number of pollen grains on stigmas among different dates determined by multiple range tests using LSD at 95% confidence on log-transformed data.

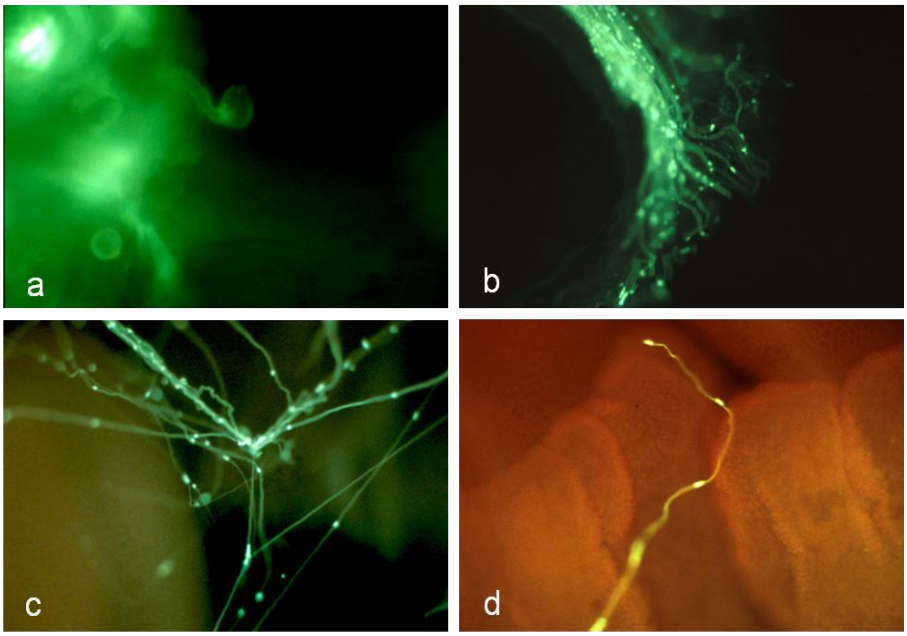

FIGURE 3. Microphotographs of pollen tubes of *Paeonia brownii* hand self-pollinated (a-b) and autogamously pollinated (c-d). (a) Pollen grains germinating between stigmatic papillae (x 40, 1/8 s). (b) Pollen tubes penetrating the transmission tissue of the style (x 20, 1/8 s). (c) Pollen tubes visible among ovules in the ovary (x10, 1/8 s). (d) Ovary of bagged flower with a single, visible pollen tube between the ovules (x10, ¼ s).

Nectar secretion and composition

Over the entire floral lifespan of *P. brownii,* nectar was secreted only from secretory tissue in the individual lobes of the perigynous disc and not from ruptured phloem in the staminal filaments of *P. brownii* as reported by Hiepko (1964) in Eurasian peonies. Neither nectar concentration nor volume nor the number of secreting lobes on a flower differed significantly between 2004 and 2005, thus, the data were combined for further analysis. On average, out of 12 lobes per disc, 3 visibly secreted nectar which collected in the concavity at the base of each lobe (Tab. 4). The mean volume of fluid collected from an individual lobe was 7.1 µL ranging from 1.0 to 32.8 µL. The number of lobes secreting nectar partially explained the total volume of floral nectar described in a linear model (r^2 = 0.33, d.f. = 38, P < 0.001).

TABLE 3. Mean number of germinated pollen grains on the stigma and pollen tubes in the style and ovary of *Paeonia brownii* flowers that received hand cross- (HCP), self- (HSP), natural insect (IP), or autogamous (AP) pollination. Different letters denote significant differences among treatments at $P \leq 0.05$ by analysis of variance on log-transformed data (untransformed means and standard deviations are shown).

	Treat-ment	*n*	Pollen no. s.d.	Range
Stigma				
	HCP	8	80.9 ± 52.6 [a]	21 – 156
	HSP	8	80.0 ± 56.4 [a]	37 – 21
	IP	8	16.6 ± 8.4 [b]	6 – 26
	AP	9	7.7 ± 10.9 [b]	0 – 28
Style				
	HCP	8	36.8 ± 18.6 [a]	17 – 61
	HSP	8	38.1 ± 24.8 [a]	8 – 80
	IP	8	5.8 ± 6.5 [b]	0 – 17
	AP	9	2.9 ± 5.7 [b]	0 – 17
Ovary				
	HCP	8	10.3 ± 11.9 [a]	0 – 35
	HSP	8	13.0 ± 9.9 [a]	0 – 28
	IP	8	2.6 ± 5.9 [b]	0 – 17
	AP	9	0.6 ± 1.3 [b]	0 – 4

No significant difference was detected in total volume of floral disc or concentration between the female (first) and male (second) phase of the unbagged or bagged flowers (Tab. 5). Although the nectar volume of unbagged flowers was only about 25% of the volume of bagged flowers in male or female phase, the concentration changed little (Tab. 5). A linear model that shows concentration weakly related to volume (r^2 = 0.14, d.f. = 38, P < 0.05) indicates that concentration was minimally affected by volume differences (Nicolson & Thornburg 2007).

Of the two hexose sugars identified, the most abundant in the nectar was glucose. The mean concentration of glucose was 37.35 µg/µL which was 3-times the mean concentration of fructose (11.33 µg/µL). Two disaccharides were found: maltose with a low mean concentration of 2.15 µg/µL and an unidentified sugar that we presume to be sucrose. The mean concentration of this sugar was 13.8µg/µL. Because sucrose can elute virtually at the same time as lactose, a lactose standard may have interfered with its positive identification (Halket & Zaikin 2003; Madeiros & Simoneit 2007). These sugars were not qualitatively analysed by mass spectrometry (MS). However, the fructose and glucose concentrations were fitted in a multiple linear regression model in which the r^2 adjusted for 10 d.f. explained 92.4% of the variation in the concentrations of the unconfirmed disaccharide (F = 62.2, P < 0.0001).

The concentrations of sixteen amino acids detected by chromatography varied widely among the 11 samples (Tab.

TABLE 4. Floral and fruit traits of *Paeonia brownii* of sampled individuals at the Blue Mountains location in northeastern Oregon, May-June 2004. Data are presented as means (± 1 s.d).

Floral trait	Mean (s.d.)	*n*
Lobes/floral nectary	12.0 (1.0)	33
Lobes with nectar/disc	3.2 (1.4)	39
Carpels/gynoecium	4.9 (0.8)	52
Ovules/carpel	18.9 (3.1)	42
Developed follicles/gynoecium	4.1 (1.3)	24
Follicle length (cm)	4.3 (6.2)	24
Total seeds/follicle	4.0 (1.3)	24
Filled seeds/follicle	3.7 (1.6)	24

TABLE 5. Mean volume and concentration percentage of secreted nectar in *Paeonia brownii* flowers sampled at the Blue Mountains location in northeastern Oregon (2004-2005 data combined). Nectary of female- and male-phase flowers visited by insects (Open) and with insects excluded (Closed).

Nectary	Phase	Volume µL ± s.d.	Concentration % ± s.d.	*n*
Closed	Female	26.45 ± 21.99	18.35 ± 6.54	20
	Male	23.63 ± 30.24	23.66 ± 10.54	19
Open*	Female	6.75 ± 6.59	23.83 ± 11.28	12
	Male	6.23 ± 2.13	32.23 ± 11.20	13

TABLE 6. Mean concentration of 16 amino acids in nectar analysed by HPLC from flowers of *Paeonia brownii* collected May 2006 at the eastside Cascades Range in central Oregon. Shown are means and standard deviations, $n = 11$ flowers.

Amino acid	Concentration µg/µL ± s.d.
Alanine	0.0040 ± 0.0031
Arginine	0.0223 ± 0.0226
Aspartic acid	0.0038 ± 0.0028
Glutamic acid	0.0026 ± 0.0014
Glycine	0.0038 ± 0.0052
Histidine	0.0050 ± 0.0073
Isoleucine	0.0006 ± 0.0011
Leucine	0.0043 ± 0.0021
Lysine	0.0012 ± 0.0021
Methionine	0.0013 ± 0.0013
Pheylalanine	0.0008 ± 0.0006
Proline	0.0109 ± 0.0052
Serine	0.0021 ± 0.0015
Threonine	0.0092 ± 0.0043
Tyrosine	0.0005 ± 0.0008
Valine	0.0018 ± 0.0013

6). In some samples the amino acid concentration fell below detectable levels; however, 10 of the 16 amino acids were detected in all samples. The mean concentration of 16 amino acids combined was 0.074 µg/µL. The mean of the individual amino acids was 0.0046 µg/µL and ranged from 0.0005-0.0223 µg/µL. The amino acids with highest mean concentration (n = 11) were arginine, proline, and threonine (Tab. 6).

Seed production and herbivory

The number of expanded carpels with green, swollen ovaries collected from flowers in an early stage of maturity averaged about 5 and ranged from 3-7 (n = 52) with 5 also the most common number of carpels (35/52). The mean number of ovules in a carpel was 18.9 and ranged from 14-28 (n = 42) (Tab. 4)

Of follicles collected from naturally pollinated flowers, the mean number of fully developed seeds per follicle was 4 (Tab. 4). The ratio of the mean number of filled seed per follicle (3.7 ± 1.6) to the mean number of ovules per carpel (18.9 ± 3.1) was about 0.20 (Tab. 4). Although the ratio of seed/ovule was low, the great majority (92.0% ± 16.7) of the seeds were viable. Filled seeds were irregularly ovoid and varied in size ranging from 5-12 mm at their widest diameter (n = 24). The mean seed weight was 0.19 ± 0.05 g at 10% moisture content (n = 20). The number of seeds in the follicle partially explained the variation in follicle length ($r^2 = 0.43$, $P < 0.001$).

Buds and opened flowers showed evidence of herbivory by at least three species of moth caterpillars in the family Noctuidae s.l. (Hardwick 1996). Flowers that had been predated by moth larvae bore tiny (1-2 mm) holes in the calyx and corolla. Caterpillars of all three species were often found under sepals or in damaged carpels (Fig. 1 f). Winged adults reared from captured larvae were identified as *Euxoa ustula* La Fontaine, *Fishia yosemitae* (Grote) and *Heliothodes diminutives* (Grote). For all species, it is the first record of *Paeonia brownii* as host plant (M. Pogue, Smithsonian, pers. com.). Crumb (1956) records *Solidago* and *Verbascum* as the most common hosts of *F. yosemitae*. Hardwick (1996) identifies *Layia platyglossa*, *Lasthenia californica* and *Madia* spp. as food plants of larvae of *H. diminutivus*.

DISCUSSION

Flower visitors and relation to floral nectar

Paeonia brownii bears hermaphroditic, mostly protogynous flowers that exhibit floral traits consistent with allogamy and a generalist pollination system. That autogamy occurs infrequently is evident in the low incidence of pollen tubes in the style and ovary of flowers denied insect contact (Tab. 3).

Floral nectar is secreted throughout the individual flower's long (9-15 days) lifespan attracting a unique variety of hymenopteran and dipteran insects that including large wasps and flies and a variety of small-medium sized bees. The floral nectary found in *P. brownii* is uncommon in Paeoniaceae, and outside of section Onaepia, is secretory

only in the *P. delavayi* complex (sensu Hong et al. 1998). Although during the male phase the flowers produce abundant pollen, unlike the Eurasian peonies they do not attract the assemblage of pollen-foraging bee and beetle visitors, as do, for example, the flowers of *P. broteroi, P. jishanensis, P. suffruticosis* and *P. officinalis* (Luo et al.1998; Sánchez-Lafuente et al. 1999; Zhou et al. 1999; Andrieu et al. 2007) or even of its closest congener *P. californica* (Schlising 1976).

Polistine and vespine wasp queens constituted more than half of the hymenopteran visitors that carried pollen of *P. brownii*. The post-hibernating wasps in early spring actively seek nectar that is easily accessed and in large volumes (Matsuura & Yamane 1990; Kearns & Inouye 1994; Mackensie et al. 2006). The high volume and concentration of carbohydrates and amino acids in the floral nectar of *P. brownii* are nutritionally important to the post-hibernating wasp queens, which can lose up to about 40% of their body weight and up to 75% of their stored fat (Spradbery 1973; Matsuura & Yamane 1984; Baker & Baker 1986; Terrab et al. 2007). They repeatedly foraged on the flowers of *P. brownii* exclusively for nectar even as the flower's presentation changed from a semi-upright bud to a nodding, open flower over the course of anthesis (Fig. I b-d), thus behaving like facultative specialists (Richter 2000; Mackensie et al. 2006).

Wasp dominance at the Blue Mountains site may be due in part to other floral resources' availability to the endemic solitary bees, but also because the wasps themselves present a threat. Vespid wasps have a large range of arthropod prey that includes Hymenoptera and Diptera (Akre & Myhre 1992; Richter 2000). They also fiercely guard their food sources and have been documented attacking other hymenopterans including ants and smaller wasps (Kurczewski 1968; Matsuura & Yamane 1990). One example is a pinned wasp (*Polistes*) in the data collection grasping an ant's head in its tarsal claws (not shown). Even though wasps typically prey on larvae following nectar consumption (Kurczewski 1968; Domínguez et al. 1989), we never observed them preying on the identified herbivores. Nevertheless, since seed production is impacted through damage by moth larvae, wasps may also provide a defensive function for *P. brownii*.

Despite their potential to predate larval herbivores (Stamp & Bowers 1988), it is doubtful that *P. brownii* flowers co-evolved with vespid wasps because their body morphology, foraging and food defending traits do not support effective cross-pollination (Domínguez et al. 1989; Galindo-González & Ornelas 2002). Vespid wasps do not necessarily emerge and forage synchronously with anthesis of *P. brownii* flowers (Akre & Myhre 1992). When vespid females do emerge in the spring, they tend to find the closest and most convenient source of nectar (Matsuura & Yamane 1990; Liebert et al. 2006). The wasps' relation to *P. brownii* more likely may be one of opportunistic mutualism. The protection that the wasp may confer to the flower through its attraction to floral nectar would be facultative (Domínguez et al. 1989).

Criorhina caudata may forage on *P. brownii* flowers guided by olfactory cues as reported elsewhere (Proctor et al. 1996; Sutherland et al. 1999). The large *Bombus* mimic visited *P. brownii* in early April before the appearance of wasp queens which may have not yet emerged in this particular region of eastern Oregon (Akre & Myhre 1992). Later in April and up to the first week in May, *C. caudata* was a frequent and constant visitor as has been reported elsewhere (Proctor et al. 1996; Goulson & Wright 1997) primarily in the morning hours when wasps were not as active. The pollen grains collected from the pilose coat of *C. caudata* were almost all from *P. brownii*, generally >200 and had a higher rate of viability than those carried by wasps (Tab. 2). C*riorhina* species may not only fill a niche in the pollination ecology of *P. brownii* at the Blue Mountains site but appear to be the more potentially effective pollen vector.

Flowers of *P. brownii* were visited by female native bees, especially small-to-medium *Lasioglossum* (Halictidae), foraging for nectar. They almost always bore pollen grains of *P. brownii* but were observed visiting the flower often after the stigma was no longer receptive (data not shown). The heterogeneous mix of pollen grains found on these bees is evidence that they visited a variety of co-flowering bee-pollinated shrubs and herbs. Such flower inconstant behaviour could increase the likelihood of pollen contamination (Rhoades & Bergdahl 1981; Shuttleworth & Johnson 2006; Sahli & Conner 2007). However, Zhou et al. (1999) found that contamination with alien pollen had no effect on seed set of *P. jishanensis*. Further investigation may show that the bees as alternative pollinators help to "buffer" the pollination system against fluctuations in their environment (sensu Sahli & Conner 2007).

Pollen carpel interactions and fecundity

The mechanical cross- and self-pollination experiments resulted in no significant difference in pollen tubes germinating on stigmas and growing in the stylar transmission tissue indicating no apparent expression of early self-incompatability (Fig. 3, Tab. 3).

Although highly variable over several weeks of sampling, the rate of fertile pollen transferred to the carpels naturally through insect (and autonomous) pollination was generally low (Fig. 2) and may help to explain the low seed set. The number of pollen tubes reaching the style under the hand pollinations was significantly higher than by natural pollination which is in contrast to other *Paeonia* species, where supplementary pollination did not necessarily increase seed set (Luo et al. 1998; Sánchez-Lafuente et al. 1999; Zhou et al. 1999; Andrieu et al. 2007). Meiotic dysfunction as well as late-acting incompatibility may be expressed when the gametes form a zygote which would account for aborted ovules (Camp & Hubbard 1963). In fact, immature follicles taken from the Blue Mountains population showed evidence of early aborted seeds in addition to underdeveloped seeds.

Similar to other *Paeonia* congeners (Schlising 1976; Sánchez-Lafuente et al. 1999; Zhou et al. 1999; Andrieu et al. 2007), the seeds of *P. brownii* are large. A low rate of conversion from ovules into viable seed is common in large-seeded plants (Guo et al. 2000). Seed number and

development may be limited by costs of prior allocation to reproductive resources; however, no such causal evidence explained sub-maximal seed production in *P. officinalis* (Andrieu et al. 2007). Perhaps female function provides fitness for this long-lived perennial herb with less than maximal seed/ovule ratios as was presented for other *Paeonia* species (Schlising 1976; Andrieu et al. 2007). Based on the relictual vasculature of *P. brownii*, Camp & Hubbard (1963) proposed that *P. brownii* may have evolved from an ancestor that produced a large and more complex carpel and presumably more seeds. Thus evolutionary selection may have been toward fewer, larger seeds. Additional studies that examine resource and genetic influences on seed set may provide a clearer explanation.

Conclusion

Flowers of the allogamous *P. brownii*, despite about half its pollen showing sterility, retained different pollination vectors over its long flowering season through the provision of floral nectar. *Paeonia brownii* blooms in a colder environment and at higher elevations and latitudes than its California congener. Vespid wasps and large, hairy syrphid flies that tolerate cool temperatures (Edwards 1980; Matsuura & Yamane 1984; Kearns 1992) appear to dominate the pollinator function. However, other flies and female bees such as *Lasioglossum* species that carry *P. brownii* pollen could potentially function as secondary pollinators, as described by Kearns & Inouye (1994) and Kearns (2001). This study in the Blue Mountains of Oregon has shed light on what appears to be a unique pollination system in *Paeonia* that warrants further investigation at other locations within its range. How the aromatic and nutrient composition of the floral nectar attracts specific insects (Nicolson & Thornburg 2007), the influence of predatory wasps in shaping the pollinator system, and how exogenous factors influence this species' low fecundity are salient questions arising from this study that warrant investigation.

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