Flora 259 (2019) 151448

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

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Pollination biology reveals challenges to restoring populations of *Brighamia insignis* (Campanulaceae), a critically endangered plant species from Hawai'i

Seana K. Walsh[a,b,*], Richard J. Pender[b], Robert R. Junker[c,d], Curtis C. Daehler[b], Clifford W. Morden[b], David H. Lorence[a]

[a] Department of Science and Conservation, National Tropical Botanical Garden, 3530 Papalina Road, Kalāheo, HI 96741, USA
[b] Department of Botany, University of Hawai'i at Mānoa, 3190 Maile Way, Honolulu, HI 96822, USA
[c] Department of Biosciences, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria
[d] Evolutionary Ecology of Plants, Department of Biology, Philipps-University Marburg, Karl-von-Frisch Str. 8, 35043 Marburg, Germany

ARTICLE INFO

Edited by Timotheus van der Niet

Keywords:
Breeding system
Floral biology
Floral nectar
Floral scent
HPLC
Moth pollination

ABSTRACT

Understanding the reproductive biology of rare plant species is fundamental to managing their restoration. *Brighamia insignis* is a critically endangered Hawaiian lobeliad endemic to the islands of Kaua'i and Ni'ihau. The flowers appear to be adapted for moth pollination although its putative pollinator is believed to be extinct or very rare. To confirm the pollination syndrome, document the breeding system, and identify potential pollinators of *B. insignis*: 1) a suite of floral characters were examined, 2) pollination treatments were performed, and 3) diurnal and nocturnal floral visitor observations were conducted at an ex situ site on Kaua'i. *Brighamia insignis* flowers contain sucrose-rich nectar and emit a strong floral scent containing benzyl alcohol, linalool, and methyl salicylate. Pollination treatments revealed that the species is primarily outcrossing with several of the study plants also capable of low levels of selfing. However, most of the plants had low pollen production and viability. No moths and only occasional non-native insect species visited the flowers of *B. insignis*. None of these insect visitors appeared to be serving as effective pollinators. In spite of this lack of flower visitation by moths, analysis of nectar and floral scent support a moth pollination syndrome in *B. insignis*. The potential loss of pollinators suggests that restoring populations of *B. insignis* may not be feasible; human assisted cross-pollination would be necessary for fruit and seed to set, as only 1% of the control and self treatment flowers formed fruit. Therefore, this species appears dependent upon intense human management to prevent its extinction.

1. Introduction

Understanding the reproductive biology (pollination ecology and breeding systems) of rare plant species is fundamental to managing their restoration (Bond, 1994; Wilcock and Neiland, 2002; Gargano et al., 2009). This need is even more pressing for rare plant species that have specialized plant-pollinator mutualisms, as specialization may preclude the formation of novel pollination mutualisms (Campbell, 2008; Anderson et al., 2011; Aslan et al., 2012, 2013). Although autogamy (self-pollination or selfing) may provide reproductive assurance in specialized species that are pollen limited, it may lower fitness in self-pollinated (selfed) compared to cross-pollinated (outcrossed) progeny due to inbreeding depression (Stebbins, 1957; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987). Plants that are incapable of autogamy and are missing a specialized animal pollinator could potentially undergo complete loss of fruit and seed production, resulting in population declines, and potentially, population extinction (Anderson et al., 2011; Gopalakrishnan and Thomas, 2014; Wolfe et al., 2014; Cerino et al., 2015).

The flora of the Hawaiian Islands has undergone a precipitous decline since the arrival of humans to the archipelago (Sakai et al., 2002). Of the 1352 vascular plant taxa that comprise the native flora (Wagner et al., 2014), 54% (724 taxa) are considered species of conservation importance in need of focused in situ and ex situ management (Weisenberger and Keir, 2014). Thirty-one percent (424 taxa) of the native vascular plant flora is federally listed as threatened or endangered (USFWS, 2018), and an estimated 130 taxa are already presumed extinct (Wood et al., 2016). Likewise, key pollinator species and even whole pollinator guilds face dramatic declines and extinctions. For example, seven of the ten specialist nectarivorous bird species, that likely pollinated one-fifth of the flora, are extinct (Sakai et al., 2002; Banko and Banko, 2009; Gorresen et al., 2009). Further, although it is

* Corresponding author at: Department of Science and Conservation, National Tropical Botanical Garden, 3530 Papalina Road, Kalāheo, HI 96741, USA.
E-mail address: swalsh@ntbg.org (S.K. Walsh).

https://doi.org/10.1016/j.flora.2019.151448
Received 27 February 2019; Received in revised form 1 August 2019; Accepted 8 August 2019
Available online 11 August 2019

estimated that approximately 67% of the native flora is pollinated by insects (Sakai et al., 2002), our understanding of the conservation status of the 5500 endemic insect species described to date is limited (Cox and Elmqvist, 2000; Nishida, 2002; Medeiros et al., 2013). For example, fewer than 1% (28 taxa) of the described native insects are federally listed as threatened or endangered (USFWS, 2018). Despite the crucial role that pollination plays in plant reproduction, only a limited number of pollinator visitation studies involving native Hawaiian plant species have been published (Carpenter, 1976; Lammers et al., 1987; Norman et al., 1997; Gardener and Daehler, 2006; Junker et al., 2010; Pleasants and Wendel, 2010; Koch and Sahli, 2013; Aslan et al., 2014; Krushelnycky, 2014; Weisenberger et al., 2014; Sahli et al., 2016; Shay et al., 2016; Weller et al., 2017; Kuppler et al., 2017; Aslan et al., 2018, 2019; Johnson and Ashman, 2019).

Brighamia A. Gray (Campanulaceae) is one of six genera that comprise the endemic Hawaiian lobeliads (Givnish et al., 2009). The genus is unique within the lineage in that the two species are caudiciform succulents with scented salverform flowers that appear adapted for moth pollination (Lammers and Freeman, 1986; Lammers, 1989; Hannon and Perlman, 2002). By contrast, the remaining five genera are small trees, shrubs or rosettes with scentless flowers that are bird pollinated (Lammers and Freeman, 1986; Wagner et al., 1999; Pender et al., 2014). The focal species of this study, *Brighamia insignis* A. Gray, is endemic to Kaua'i and historically to Ni'ihau where it occurred on cliff habitats between sea level and 400 m elevation (Lammers, 1999; Wagner et al., 1999). The species is listed as Endangered by the United States Fish and Wildlife Service (USFWS, 2017) and assessed on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species as Critically Endangered (Possibly Extinct in the Wild) (Walsh, 2016). Only a single wild plant may remain on the Nā Pali Coast of Kaua'i, last seen in 2012 (K. Wood, National Tropical Botanic Garden [NTBG], personal communication). Hurricanes, landslides, invasive plant and animal species (particularly goats that eat the plants and disturb their cliff habitats), and the possible loss of a putative obligate moth pollinator are believed to be the main factors that have led to the decline of *B. insignis* (USFWS, 2007).

Despite the conservation status of *B. insignis*, significant knowledge gaps concerning the reproductive biology of this species exist. Lammers (1989), in a taxonomic revision of the genus, briefly mentioned that the nectar of *B. insignis* is sucrose rich, but did not provide supporting data. Kaiser (2010) analyzed the scent volatiles produced by *B. insignis* flowers finding that they emit compounds typically associated with moth-pollinated plants. However, the flowers were sampled only during the day. Other studies have recorded distinctions between day and night volatile compound emission rates in moth-pollinated plants, with higher emission rates in the evening (Raguso et al., 2003; Dötterl et al., 2005; Hoballah et al., 2005; Peter et al., 2009; Martinell et al., 2010; van der Niet et al., 2015). In addition, conservation practitioners who have worked with cultivated *B. insignis* plants have found that the species either sets no or limited fruit, and there is an increase in fruit and seed set when flowers are hand pollinated with outcross pollen (A. Trask, NTBG, personal communication). This suggests that *B. insignis* may be predominantly outcrossing and pollen limited. Other aspects of the reproductive biology of *B. insignis* remain unknown.

To improve our understanding of reproduction in *B. insignis*, we undertook three separate lines of investigation. First, to better understand the pollination syndrome, we collected quantitative data on a suite of floral traits. Second, cultivated plants were used to identify the breeding system. Third, a floral visitation study was undertaken to identify potential extant pollinators at an ex situ population planted approximately 2 km from the known historical range of this species (K. Wood, NTBG, personal communication).

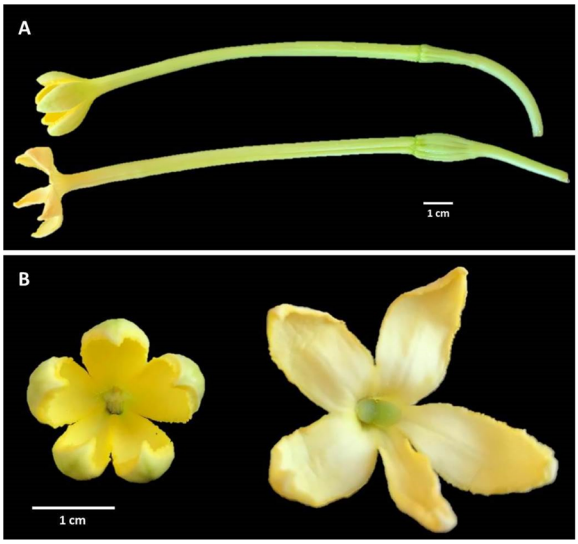

Fig. 1. *Brighamia insignis* flowers in male phase (top A and left B) and female phase (bottom A and right B).

2. Materials and methods

2.1. Study species

Brighamia insignis is a caudiciform succulent that grows up to 5 m tall (Wagner et al., 1999). Plants are typically single-stemmed, although the stems may branch in rare cases (K. Wood, NTBG, personal communication). The flowers are erect and held in 3–8 flowered axillary racemes (Lammers, 1989). Throughout the study period, flowering primarily occurred between June and December, with peak flowering during September and October and occasional flowers at other times of year (S. Walsh, personal observation). The salverform flowers range in length from 7 to 14 cm and are yellow to pale cream or rarely white (Fig. 1; Lammers, 1989). Like all Hawaiian lobeliads, flowers are protandrous (hermaphrodite flowers that pass first through a male phase followed by a female phase) (Lammers, 1989, 1999). During the male-phase, pollen is shed from the connate anthers as the style elongates through the staminal column. Flowers enter the female-phase once the style exerts and the stigmatic lobes expand.

2.2. Floral biology

2.2.1. Floral measurements

Fourteen male-phase flowers (1–4 flowers per plant from seven different plants) and fifteen female-phase flowers (1–3 flowers per plant from 10 different plants) were collected from the NTBG Conservation and Horticulture Center, McBryde Garden, Kaua'i in May 2011 and at Queen Kapiolani Garden (QKG) in Honolulu in June 2011 and February 2012. The NTBG living collections utilized were primarily of Ho'olulu Valley origin, which is located on the Nā Pali coast of Kaua'i. Some individual plants were grown from wild collected seed and others were from F1 and F2 generation crosses. Some individuals lacked provenance data. The voucher specimen, *David Lorence* 10438 (Herbarium PTBG), is representative of the NTBG living collection. The plants at QKG were of unknown origin. The following three measurements were made on each flower: 1) corolla tube length (excluding lobes), 2) corolla tube width midway along the length of the corolla tube, and 3) width at the top of the corolla tube, immediately below the corolla lobes. A flexible plastic ruler was used to record all measurements to the nearest half millimeter and the mean was calculated for each of the three floral measurements.

2.2.2. Nectar standing crop

In the summer of 2011, 15 cultivated *B. insignis* plants grown from

seed (E. Romanchak, Native Nursery LLC, personal communication) were purchased from the Native Nursery LLC based in Kula, Maui and shipped to Oʻahu. These cultivated plants lack provenance data. Seven male-phase (1–2 flowers per plant from four different plants) and 12 female-phase (1–2 flowers per plant from six different plants) flowers of this stock were harvested and maintained in an upright position to prevent nectar spillage. Within two hours after collecting, the corollas of all flowers were partially dissected by hand. The nectar was collected and volume determined using a calibrated micropipette (0–200 μL). Mean nectar volume per flower was subsequently calculated. Nectar samples were stored in 1.5 mL microcentrifuge tubes for immediate percent mass sucrose measurements and then subsequently stored at −20 °C for future high-performance liquid chromatography (HPLC) analysis (summarized in Section 2.2.4).

2.2.3. Position of nectar in flowers

To estimate the proboscis length that a potential insect visitor would need to access the nectar in male- and female-phase flowers, the distances between the upper reaches of nectar in the corolla tube and the 1) apex of the anther in male-phase flowers, and 2) the anther apex and stigma surface in female-phase flowers was measured in the same flowers that were used to measure nectar standing crop. The corollas were partially dissected by hand to expose the nectar within the corolla and distances measured to the nearest half millimeter using a flexible plastic ruler.

2.2.4. Nectar sugar composition

Using the same flowers from which nectar standing crop was measured, a handheld refractometer (model: Eclipse 0–50% weight/volume [w/v]; Bellingham and Stanley Ltd, Basingstoke, UK) was used to quantify the percent mass sucrose in the nectar of each flower. Mean sucrose concentration was subsequently calculated for all the sampled flowers. HPLC analysis was later conducted in the Department of Tropical Plant and Soil Sciences, University of Hawaiʻi at Mānoa (UHM), using a Shimadzu Model 20 HPLC with a CBM-20A controller, LC-20AT pump, SIL-20A automatic injector, CTO-20A column oven and a ELSD-LT-II Evaporative Light Scattering Detector (Shimadzu Corp., Kyoto, Japan) with a Fast Carbohydrate Analysis Column (100 mm × 7.8 mm) and precolumn (Bio-Rad Laboratories, Hercules, California, USA). The methods followed those of Pender et al. (2014) to identify and quantify the proportions of sugars (sucrose, glucose, and fructose) in the nectar samples.

2.2.5. Floral scent

Floral scent samples were collected from an individual *B. insignis* cultivated plant at NTBG in December 2013. The scent of two open flowers on the same plant, one in an early male phase and one that was in a later female phase, was collected using the dynamic headspace sampling method (Kuppler et al., 2017; Junker and Larue-Kontić, 2018). Scent was sampled once before sunset (16:00) and once after sunset (19:30) by enclosing the flowers in scentless oven bags. Headspace (air surrounding the flowers) was enriched for 40 and 53 min (time varied slightly for each sample) and the scented air was sucked through volatile traps for 2 min by a membrane pump with a flow rate of 200 mL/min. Volatile traps contained a mixture of 1.5 mg Tenax-TA (mesh 60/80; Supelco, Germany) and 1.5 mg Carbotrap B (mesh 20/40; Supelco, Germany). Volatiles were desorbed from traps using an automatic thermal desorption (TD) system (model: TD-20; Shimadzu Corp., Kyoto, Japan).

Samples were analyzed at the University of Salzburg by coupled gas chromatography (GC) and mass spectrometry (MS) (model: QP2010 Ultra EI; Shimadzu Corp., Kyoto, Japan). The GC was equipped with a 60 m long column (Zebron ZB-5; Newport Beach, California, USA) with an inner diameter of 0.25 mm and a film thickness of 0.25 μm. The column flow of a helium carrier gas had a rate of 1.5 mL/min. Gas chromatography oven temperature was kept constant at 40 °C for 1 min, then increased by 6 °C/min until a maximum temperature of 250 °C was reached. The MS interface and ion source were set at 260 °C and 200 °C, respectively.

For identification of the scent compounds, GCMS solutions Software Version 2.72 (Shimadzu Corp., Kyoto, Japan) was used by comparing mass spectra with authentic standards, computer libraries (ADAMS, ESSENTIALOILS-23 P, FFNSC 2, W9N11) and Kovats indices generated using n-alkanes.

2.2.6. Phenology of individual flowers

Eight flowers in bud were selected at random from among four different plants and monitored over the course of their lifespan at the NTBG McBryde Garden between May 17 and May 25, 2011. Occurrence of male (pollen presentation) and female (stigmatic) phases were monitored and noted each morning (between 07:30 and 10:30) and each evening (between 15:30 and 18:30) on tagged flowers. Mean time (in hours and days) spent in each of the flower phases was subsequently calculated for all flowers. Flowers were considered to have transitioned from male-phase to female-phase once the two rounded stigmatic lobes were fully expanded and partially reflexed. Flowers were considered senesced when the stigma became flaccid and the corolla browned.

2.3. Breeding system

2.3.1. Pollination treatments

To assess if pollinators are necessary for fruit and seed production in *B. insignis*, manipulative pollination treatments were conducted on cultivated plants over the course of three years. In 2011, pollination treatments were conducted on the plants from Native Nursery LLC that flowered in November and December of 2011 and August through November of 2012. In 2012, four additional plants were purchased from Leeward Community College (LCC), Oʻahu, and three plants were obtained from the Department of Plant and Environmental Protection Sciences (PEPS), UHM. All plants were kept on an outdoor shaded balcony in the St. John Plant Science building on the UHM campus for the duration of the study. In 2011, pollen donors for the outcross treatment were from plants growing at the QKG in Honolulu. In 2012, the plants at QKG had died, so pollen for the outcross treatment was from different sources (e.g., plants from Native Nursery LLC, LCC, and PEPS). In June, September and October of 2013, 32 cultivated plants representing 10 different NTBG accessions were utilized for pollen manipulations in the Conservation and Horticulture Center at the NTBG McBryde Garden on Kauaʻi.

Manipulative pollinations consisted of a control and the following three treatments: self (no pollen added), self plus (addition of pollen from another flower on the same plant), and outcross (addition of pollen from a different plant). The self treatment tested if the plants are capable of autonomous self-pollination. The self plus treatment tested if the plants are capable of geitonogamy (self-compatibility between different flowers of the same individual plant). Flowers in the self treatment were bagged while in bud to exclude potential pollinators (although the plants used for pollination treatments were not near a native forest or the historic range of *B. insignis*) using 15 cm × 4 cm bridal veil material bags (1 × 1 mm mesh) that were held in place with zip ties fastened around the pedicel and retained on the flowers until they senesced. Flowers in the self plus treatment were bagged while in bud as for the self treatment and monitored until stigmas were receptive. Once stigmas were receptive, the connate anthers from a male-phase flower of the same plant were removed, longitudinally dissected and pollen was gently dabbed on the stigmas. The flowers were subsequently re-bagged. An outcross treatment was used as a control against which to contrast the self and self plus treatments. The same methods were followed as that of the self plus treatment except pollen from a male-phase flower of a different accession and/or source was used. Flowers in the control were unmanipulated and unbagged (potential pollinators not excluded). All flowers were labeled with jewelry tags. Appendix A

provides a breakdown of the number of flowers and plants used in the control and each manipulative pollination treatment during each season (2011, 2012, and 2013) and in all seasons combined. All mature fruits were cut open using a razor blade, and then seeds were extracted using a dental pick and counted. Mean seed counts were subsequently calculated for the control and each treatment.

We used the following indices and cut-off points provided by Rodger and Ellis (2016) to classify the breeding system: index of self-incompatibility (ISI) (self-incompatible if ISI ≥ 0.8) and autofertility index (AFI) (pollinator dependent if AFI < 0.2). The two indices are calculated as follows: ISI = 1 – (% hand-self fruit formation/% hand-cross fruit formation); AFI = % autonomous self fruit formation/% hand-cross fruit formation (Rodger and Ellis, 2016).

2.3.2. Pollen viability

Pollen viability was tested in September, October and November 2013 in a subset of plants at NTBG that were used for pollination treatments that season. Pollen was collected from flowers in late bud or during the male-phase, stored in 1.5 mL microcentrifuge tubes, and stained within 2.5 h of collecting using a modified Alexander's stain technique (Alexander, 1969) developed by Peterson et al. (2010). However, unlike Peterson et al. (2010), samples were not fixed prior to staining and the stained pollen for this study was not heated, as trials indicated no distinguishable differences in pollen staining between heated and non-heated samples (S. Walsh, personal observation). Pollen viability was determined by counting pollen grains that stained magenta-red (viable) compared with grains that stained blue-green (non-viable) using a compound light microscope (Zeiss Primo Star, Carl Zeiss Microscopy, Jena, Germany). In all but one case, three-hundred pollen grains total were counted from each plant sampled, while one plant had all 356 pollen grains counted. Mean percentage pollen viability for all the samples was subsequently calculated.

2.4. Floral visitors

All floral visitor observations were undertaken with outplanted individuals at the NTBG Limahuli Garden and Preserve in Hā'ena on Kaua'i (22.21914°, 159.5758°; from 26 to 84 m elevation), as natural populations are no longer extant. The site receives approximately 2560 mm of rainfall per year (Giambelluca et al., 2013) and is situated approximately 2 km from former natural populations of *B. insignis* (K. Wood, NTBG, personal communication).

Floral visitor observations took place over six days in September and October 2013 (18 plants) and three days in September 2014 (13 plants). Diurnal floral visitor observations took place between 06:50 and 19:20. Nocturnal observation hours started at civil dusk which, depending on the date, started as early as 18:45, and were conducted until as late as 23:55. At night, a headlamp with red light was used to monitor potential pollinators. All flowers on an individual plant were first counted and monitored from within two meters of the focal plant during 15-minute observation periods. Depending on the day, between eight and 31 (mean = 23) 15-minute observation periods occurred each day. During the observation period, the type, frequency and fate of visit (e.g., sexual organ[s] contact versus non-sexual flower parts [e.g., corolla lobes or tube]) of any organisms visiting *B. insignis* was recorded. Floral visitors were photographed with an Olympus Tough TG-1iHS 12 megapixel waterproof digital camera (Tokyo, Japan) with optical zoom and later identified by professional entomologists from the Bernice Pauahi Bishop Museum, Hawai'i Department of Agriculture, and the University of Hawai'i.

2.5. Data analysis

When comparing the 1) nectar standing crops between male- and female-phase flowers, 2) nectar sucrose concentrations between male- and female-phase flowers, and 3) length of time spent in the male and female phases between flowers, the data were first tested for equal variances using Anderson Darling Normality tests. Upon verification of parametric assumptions, a two-sample *t*-test was used to compare means. Measurements of the distance between the maximum nectar level and the reproductive organs in male- and female-phase flowers were log transformed ($\log_{10}$), tested for equal variances as for the data above, and a two-sample *t*-test used to test for differences in the means between the variables.

Owing to a limited number of flowers produced per plant and the small total number of fruits that formed, it was not possible to test for statistical differences among individual plants in pollination treatment responses; therefore, statistical analyses were done using flowers as the unit of replication rather than plants (Cory et al., 2015). We used a χ^2 test to determine if there were significant differences in the proportion of flowers that formed fruit among the control and three pollination treatments, while a two-sample *t*-test was used to compare the number of seeds per fruit in the outcross treatment with seeds produced per fruit in the other three treatments combined. Statistical analyses were undertaken in Minitab version 17 (Minitab Inc., State College, Pennsylvania, USA) and significance was accepted at an alpha (α) level of 0.05. All means are presented ± one standard deviation (SD).

3. Results

3.1. Floral biology

3.1.1. Floral measurements

Mean corolla tube length (excluding lobes) of *B. insignis* flowers was 102.7 ± 14 mm. Mean corolla tube width midway along the length of the corolla tube was 5.3 ± 0.4 mm. The mean width at the top of the corolla tube, immediately below the corolla lobes, was 6.6 ± 0.5 mm.

3.1.2. Nectar standing crop

The mean nectar standing crop of all flowers sampled, both female-phase and male-phase flowers combined, was 94.6 ± 66.1 μL. Female-phase flowers contained significantly more nectar than male-phase flowers (female = 136.8 ± 43 μL; male = 22.4 ± 8.9 μL; t = 8.9; df = 12; P < 0.001).

3.1.3. Position of nectar in flowers

Mean distance between the maximum nectar level and anther apex in male-phase flowers was 54.4 ± 13.9 mm. The mean distance between the uppermost position of nectar and the anther apex and stigma surface in female-phase flowers was 26.2 ± 16.0 mm and 35.2 ± 16.0 mm, respectively. There was no significant difference (t = 1.38; df = 21; P = 0.18) when comparing the distance between maximum nectar level and anthers (26.2 ± 16.0) and stigmas (35.17 ± 16.0) in the female-phase flowers that were measured. However, there was a significant difference (t = 2.65; df = 14; P = 0.02) when the distance between the maximum nectar level and the anthers in male-phase flowers (54.4 ± 13.9) was compared with the maximum nectar level and the stigma surface (35.2 ± 16.0) in female-phase flowers.

3.1.4. Nectar sugar composition

The mean percentage of sucrose was 8.0 ± 1.5%. There was no significant difference (t = 1.4; df = 16; P = 0.19) in sucrose percentage between male-phase (8.5 ± 0.9%) and female-phase (7.7 ± 1.7%) flowers. HPLC results showed a mean sucrose to hexose ratio (% sucrose)/(% fructose + % glucose) of 0.9. Nectar samples were rich in sucrose (46.1 ± 7.9%) and fructose (43.9 ± 5.9%), but contained only small amounts of glucose (10.0 ± 2.7%).

3.1.5. Floral scent

In total, 14 volatile organic compounds (VOCs) were detected in the floral scent of *B. insignis*, 13 of which were identified by comparing their

Table 1
Day and night proportional emission rates of all volatile organic compounds (VOCs) in *B. insignis* floral scent.

Family or Class of Compounds	Volatile organic compound (CAS number)	Kovat's retention index	Proportion during day	Proportion at night
Aliphatics	(Z)-3-Hexen-1-ol (928-96-1)	855	0.015	0.009
	1-Hexanol (111-27-3)	867	0.021	0.016
	(Z)-3-Nonenol (10340-23-5)	1156	0.003	0.006
	Decanal (112-31-2)	1208	0.002	0
	Heptyl butyrate (5870-93-9)	1290	0	0.008
Aromatics	Benzyl alcohol (100-51-6)	1040	0.466	0.545
	Methyl salicylate (119-36-8)	1205	0.021	0.011
Monoterpenes	delta-3-Carene (13466-78-9)	1018	0.003	0.002
	1,8-Cineole (470-82-6)	1046	0	0.005
	(Z)-Linalool oxide furanoid (5989-33-3)	1079	0.001	0.001
	Linalool (78-70-6)	1103	0.412	0.349
Nitrogen-containing	Indole (120-72-9)	1305	0.026	0.026
Sesquiterpenes	(E)-Nerolidol (40716-66-3)	1571	0.029	0.020
Unknown	Unknown, m/z: 32, 57, 41, 43, 111	1507	0	0.002
Sum [ng h-1]			5801	7038

mass spectra and retention times with those of standard substances. The remaining detected VOC could not be identified. The two dominant volatiles were benzyl alcohol and linalool. Benzyl alcohol made up an average of 47% and 54% of the total volatile compounds identified in the floral scent samples during the day and at night, respectively. Linalool made up an average of 41% during the day and 35% at night of total emissions. Proportional emission rates during the day and at night of all volatiles detected are listed in Table 1.

3.1.6. Phenology of individual flowers

Flowers remained open between five and seven days ($x = 6.2 \pm 0.6$ days). Mean time spent in the male-phase (38.8 ± 10.6 h; ca. 1.6 days) was significantly shorter ($t = 8.69$; $df = 10$; $P < 0.01$) than the mean time spent in the female-phase (109.6 ± 20.5 h; ca. 4.6 days).

3.2. Breeding system

3.2.1. Pollination treatments

Combining results of the three flowering seasons during which the breeding system study was conducted, 81% of the fruits produced (13 out of 16 fruits) and 95% of the seeds produced (4237 out of 4473 seeds) were in the outcross treatment. Among flowers in the outcross treatment, 33% developed fruits, whereas all but one fruit in each of the control, self and self plus treatments aborted shortly after the flowers senesced, corresponding to fruit development rates of 1%, 5%, and 1%, respectively, averaged across the three years that the study was undertaken. There was a significant difference in the proportion of fruit formed between the pollination treatments ($\chi^2 = 11.03$, $df = 3$, $P = 0.05$). However, all 13 outcrossed fruits were produced in 2011 and all outcrossed plants in 2011 produced at least one fruit; whereas, no mature fruits formed in the outcross treatment in 2012 and 2013. Formation of the single mature fruit for the self plus treatment occurred in 2012 and one each for the control and self treatment in 2013. The two individual flowers that formed fruits in the selfing treatments were from different plants, and other flowers on those same plants failed to produce fruits following selfing treatments; these sparse data do not allow statistical linking of selfing to individual plants. The ISI was 0.85 and the AFI was 0.03.

There were also differences observed in the number of seeds formed per fruit among the control and pollen manipulation treatments (Fig. 2). The single fruits that formed in the control, self and self plus treatments contained 142, 41 and 53 seeds, respectively; whereas, the thirteen fruits that formed in the outcross treatment produced higher mean seed counts (326 ± 115) compared to the control and two other treatments combined ($t = 3.45$, $df = 14$, $P = 0.002$).

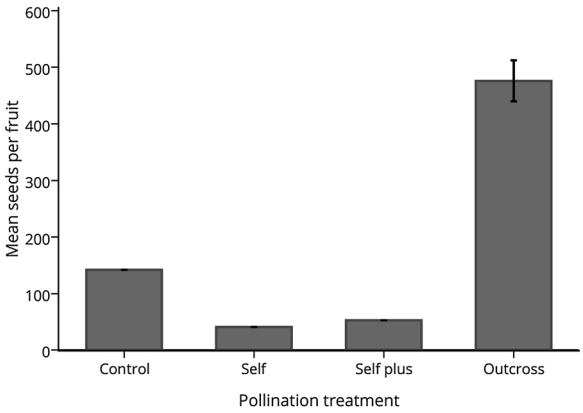

Fig. 2. Mean (± SE) seed counts among successfully developed fruits for the control and three pollination treatments applied to *B. insignis* plants. Note: no error bars are present for the control, self and self plus treatments as only one fruit formed in each treatment.

3.2.2. Pollen viability

Average *B. insignis* pollen viability in 2013 was 4.7 ± 0.2% (see Appendix B for percent viability of each flower examined and Appendix C for all NTBG accessions and plants used to test pollen viability). The non-viable pollen grains not only stained blue-green, an indication of non-viability, but also appeared shriveled and smaller compared to those that stained pink (considered viable).

3.3. Floral visitors

Diurnal and nocturnal observation hours totaled 29.5 and 21.5, respectively. A total of six different non-native invertebrate species contacted the floral reproductive organs of *B. insignis* flowers during the observational study. Half of the insect visitors were non-native ants (*Brachymyrmex obscurior, Ochetellus glaber*, and a third unknown ant species), while the remainder were an unidentified fly (Order Diptera), a thrips (Order Thysanoptera) and possibly a beetle (Order Coleoptera). The mean duration of time that these floral visitors contacted the anthers on both male- and female-phase flowers was 6.25 ± 6.7 s. Mean duration of time spent visiting stigmas of female-phase flowers was 31.6 ± 51.56 s.

Collectively, the six invertebrate species made nine visits to *B. insignis* flowers, contacting one flower during each separate visit. Five of the visits occurred during the day and four occurred at night. Diurnal visitors included *Ochetellus glaber*, an unidentified species of ant (Family Formicidae), a fly (Order Diptera) and a thrips (Order Thysanoptera). Nocturnal visitors included *Brachymyrmex obscurior*, the same

unidentified species of ant as the unidentified diurnal ant visitor, and possibly a beetle (Order Coleoptera). Four of the visitors contacted stamens, while five contacted stigmas. No insect visitor contacted both stamens and stigmas during any single visit.

Over the course of the study, fewer than 10% of available flowers per day were visited by an insect that came into contact with the stigma or stamens. Across days, the average percent of available flowers that were visited and resulted in floral reproductive organ contact was 1.1 ± 2.0% per hour during the day and 1.2 ± 2.0% per hour at night. An average of 2.7 ± 4.9% of the plants were visited per hour during the day and 5.0 ± 11.0% per hour at night.

4. Discussion

4.1. Floral biology

Although some studies have demonstrated that pollination syndromes are not always reliable in predicting the functional pollinator group of a plant species (Hingston and McQuillan, 2000; Ollerton et al., 2009) and we did not observe moths visiting flowers during our observations, the floral traits of *B. insignis* that we examined are consistent with traits of other plant species found to be visited and pollinated by moths. It is likely that *B. insignis* flowers are adapted for moth, and possibly hawkmoth (Sphingidae), pollination, as has been suggested by Lammers and Freeman (1986).

Floral traits of *B. insignis* consistent with moth pollination include the differences in flower morphology (e.g., flower shape and corolla tube length) between *Brighamia* and the other five Hawaiian lobeliad genera that have shorter, curved corollas that are bird pollinated (Lammers and Freeman, 1986; Lammers, 1999). Based on phylogenetic reconstructions, the Hawaiian lobeliads are believed to have evolved from a single bird-pollinated ancestor approximately 13 million years ago (Givnish et al., 2009). *Brighamia* likely underwent a shift to moth pollination in less than 3.4 million years after colonization (Givnish et al., 2009). This timeframe is comparable to other genera that have undergone evolutionary shifts in their pollination syndromes over relatively short geological time frames (Whittall and Hodges, 2007; Bastida et al., 2010; Lagomarsino et al., 2016).

Plant species with moth pollinated flowers typically possess white or pale colored corollas that are easier for moths to see at night (Fenster et al., 2004). The adaxial corolla lobe color of *B. insignis* flowers, when initially opening, is bright greenish yellow to brilliant yellow, eventually fading to white during senescence (S. Walsh, unpublished data). The transition of *B. insignis* corolla lobes from yellow to white may have evolved to either attract pollinators before the flower senesces or to signal to pollinators that the flower no longer contains floral rewards (Gori, 1983). Due to the limited insect visitors in our pollinator study, we could not test whether visitation is influenced by the different phases of corolla color in *B. insignis*.

There are very few published studies documenting moth pollination in Hawai'i (Norman et al., 1997; Weller et al., 2017). Observations by Norman et al. (1997) revealed native pyralid moths as the most common floral visitors to *Schiedea lydgatei* on Moloka'i. Weller et al. (2017) found that a native moth (*Pseudoschrankia brevipalpis*) is an effective pollinator of *S. kaalae* and *S. hookeri* on O'ahu. Based on floral traits and floral visitor observations, three graduate thesis and dissertation projects have speculated moth pollination in Hawaiian *Hibiscus* (Huppman, 2013), *Lysimachia* (T. Kroessig, Lyon Arboretum, personal communication) and *Scaevola* (Elmore, 2008). It is possible that the paucity of documented moth pollination is due to the decline of populations of native moth pollinators in Hawai'i due to habitat loss and degradation since human colonization. However, to date, no studies have directly linked the loss of moth pollination with plant population declines in Hawai'i, as has been shown elsewhere (Johnson et al., 2004).

The mean nectar standing crop of *B. insignis* flowers examined in this study (both male-phase and female-phase combined) was higher than the recorded ranges in other presumed and observed hawkmoth (Sphingidae) pollinated plant species (Wolff, 2006; Martins and Johnson, 2007, 2013; Paulino-Neto, 2013; Mitchell et al., 2015; Johnson et al., 2017). The larger nectar volumes recorded in *B. insignis* flowers could potentially be due to one or a combination of factors. These include the size and foraging demands of the historic pollinators, the recent evolution of *B. insignis* from bird pollinated ancestors that are known to produce large nectar volumes (Lammers and Freeman, 1986; Givnish et al., 2009; Pender et al., 2014), or the fact that *B. insignis* have larger flowers than most of the plant species measured in the aforementioned studies.

The greater quantity of nectar in female-phase flowers is either due to the accumulation of nectar between the flower phases or a bias towards greater nectar production during the female phase. If female-phase flowers produce more nectar, this trait may have evolved to ensure pollinators spend adequate time at female-phase flowers, thereby enabling the deposition of pollen on stigmas, as has been shown in other plant species (Langenberger and Davis, 2002; Carlson and Harms, 2006).

The ability of potential pollinators to successfully forage for nectar from *B. insignis* flowers is partially dictated by the uppermost position of nectar within the corolla tube. Our results indicate that a moth, for instance, would need a longer proboscis to access nectar in male-phase (54.4 ± 13.9 mm) when compared to female-phase (35.2 ± 16.0) flowers. This disparity in nectar production volume between male and female flowers may be adaptive; moths with short proboscises, who struggle to reach nectar in male-phase flowers, may end up with larger pollen loads. Upon visiting female flowers these same insects with large pollen loads may increase likelihood of pollination and by being rewarded with ample and easy to access nectar, help ensure return visits to flowers in general. Unfortunately, the lack of insects visiting the flowers of *B. insignis* in our study did not allow this hypothesis to be tested.

There are four hawk moth species (Sphingidae) native to Hawai'i (Zimmerman, 1958, 1978; Nishida, 2002; W. Haines, UHM, personal communication). The proboscis lengths of these hawkmoth species are unavailable, making it difficult to predict the potential hawkmoth pollinator(s) that historically may have visited *B. insignis* flowers. However, based on the position of nectar in male- and female-phase flowers recorded in our study, proboscis lengths in the 974 species of smaller, non-hovering native Hawaiian moths are likely too short to reach the nectar in *B. insignis* flowers (Zimmerman, 1958, 1978; Nishida, 2002; W. Haines, UHM, personal communication).

Following the nectar-sugar classification of Baker and Baker (1983), we found that *B. insignis* produces nectar that is sucrose-rich, which is associated with pollination by moths, butterflies, long-tongued bees, and hummingbirds (Lammers and Freeman, 1986). Long-tongued bees and hummingbirds are not part of the native Hawaiian fauna (Zimmerman, 1948; Lammers and Freeman, 1986). Nectar of the other Hawaiian lobeliad genera, in contrast, is hexose-rich, which is associated with bird pollination (Lammers and Freeman, 1986; Pender et al., 2014).

Benzyl alcohol and linalool made up a large proportion of the volatile emissions of *B. insignis* flowers. Both of these VOCs have been described as emitted by flowers of plant species that are visited and pollinated by moths (Jürgens et al., 2002; Hoballah et al., 2005; Dobson, 2006; Riffell et al., 2009, 2012). Riffell et al. (2009) found that benzyl alcohol and linalool elicited flight and foraging behavioral responses in a hawkmoth known to visit a moth pollinated plant species. However, it should be noted that both volatiles are also commonly emitted by plant species that are not moth pollinated (Knudsen et al., 2006). The fact that benzyl alcohol was the most abundant VOC emitted by *B. insignis* flowers (47% and 54% of total VOCs in the two floral scent samples), and that the emission rate was higher at night compared to during the day, lends further support to the supposition that *B. insignis*

evolved with a moth as its primary pollinator. By contrast, Kaiser (2010) found that (E)-nerolidol (41% of total VOCs) had the highest emission rate in the *B. insignis* that he sampled. Although the proportions of VOCs differed slightly between Kaiser's (2010) and our study, overall, the VOCs detected were similar.

Brighamia insignis flowers remained open for time periods comparable to other dichogamous species (Gao et al., 2015), including bird-pollinated Hawaiian lobeliad species (R. Pender, unpublished data). Within *B. insignis* flowers, temporal sex allocation is female biased, with flowers spending 2.8 times longer in the female-phase compared to the male-phase. An extended female-phase may have evolved to ensure adequate time for pollination in a variable pollinator environment (Stratton, 1989). Alternatively, longer female-phases may allow sufficient time for pollen tubes to grow down the relatively long style to fertilize ovules. However, we did not measure pollen tube growth rates in *B. insignis*. Among other species, pollen tube growth rates are known to vary widely, ranging from 0.06 to 20 mm per hour (Tangmitcharoen and Owens, 1997). Finally, four bird-pollinated Hawaiian lobeliads (R. Pender, unpublished data) and *B. insignis* have similar patterns of temporal sex allocation despite the difference in pollinator guilds. Given the relatively recent evolution of Hawaiian lobeliads (Givnish et al., 2009), whole flower and within-flower sex duration may be phylogenetically constrained in the lineage (e.g., Stratton, 1989).

4.2. Breeding system

Although there were inconsistencies in fruit formation in the breeding system study between years, overall, the results suggest that *B. insignis* is primarily outcrossing but occasionally capable of low levels of selfing as part of a mixed-mating strategy. Following the cut-off points for breeding system classification by Rodger and Ellis (2016), *B. insignis* is categorized as pollinator-dependent self-incompatible (SI). The exact timing and mechanism of selfing in *B. insignis* requires further investigation. Although bird-pollinated Hawaiian lobeliads also employ a mixed mating system (Aslan et al., 2014; R. Pender, unpublished data), *B. insignis* appears to have lower rates of autogamy compared to the other Hawaiian lobeliad species studied to date. Selection may have favored outcrossing in *B. insignis*, as inbreeding depression has been shown to be severe in species adapted for xeric habitats (Fox and Reed, 2010). However, our breeding system results should be interpreted with some caution, as they are complicated both by the limited production of pollen that was mostly non-viable and by the study utilizing different plants and occurring in different locations between years. Furthermore, the control treatment in our study in which flowers were exposed to potential pollinators, is a very limited assessment of insect pollination and pollen limitation because the plants used were not located near native habitats or the historic range of *B. insignis*.

Pollinator loss has been cited as one of the potential reasons for the decline of *B. insignis* (USFWS, 2007). Our results support this supposition; without insect assisted outcrossing, *B. insignis* fruit and seed production, and subsequent seedling recruitment, would have likely been low in natural or outplanted populations.

Our pollen viability analysis suggests that some *B. insignis* plants may have low pollen production and viability. The plants used in 2013 for pollen manipulations had low viability, potentially affecting fruit formation. Interestingly, both of the single fruits that did form in the control and self treatment in 2013 occurred on a plant that produced viable pollen. Observations by Gemmill et al. (1998) indicated that only ca. 5% of flowers in natural populations and garden collections of both *B. insignis* and *B. rockii* produced pollen. By contrast, fruits were produced abundantly from outcrossing in 2011, suggesting that pollen viability was likely high in the plants that were used as pollen donors. Unfortunately, the breeding system experiments were not carried out on these pollen donor plants.

There are at least three possible reasons why the majority of the plants utilized in this study were producing low amounts of pollen. First, it may be due to an environmental response. Several studies have found that changes in temperature, relative humidity, and nutrient availability have an effect on development of pollen (Lau and Stephenson, 1993; Astiz and Hernandez, 2013; Mercuri et al., 2013; Donders et al., 2014; Flores-Rentería et al., 2018). For example, Flores-Rentería et al. (2018) found that *Pinus edulis* pollen viability was negatively affected by high temperatures at both the dispersal and germination stages of pollen development, with a larger effect at the germination stage. Cultivated plants in the NTBG Conservation and Horticulture Center nursery that were used for the breeding system study in 2013 were growing in a warmer environment, in a greenhouse at a lower elevation with less wind and rainfall, compared to that of their natural habitat. Second, some *B. insignis* plants tend toward female function (either gynomonoecious or monoecious; Gemmill et al., 1998). This is based primarily on field observations of wild populations, in which approximately two-thirds of plants never produced pollen (i.e., functionally female); however, when out-cross pollen was applied to the stigmas of these plants, many fruits, containing abundant viable seeds, were formed (S. Perlman, Plant Extinction Prevention Program [PEPP], personal communication). Third, inbreeding might reduce pollen production (Good-Avila et al., 2003; Hayes et al., 2005). For example, Good-Avila et al. (2003) found decreases in pollen production per flower as the level of inbreeding increased in *Campanula rapunculoides*. Cultivated *B. insignis* have likely undergone severe inbreeding, with most of those currently in cultivation at NTBG the result of crosses among original collections from Hoʻolulu and Waiahuakua populations (NTBG, 2018). The reason for the low pollen production observed in our study and by others warrants further, more detailed, investigation.

4.3. Floral visitors

Based on floral visitor observations, it appears unlikely that the observed insect visitors are effectively pollinating *B. insignis* outplantings at Limahuli; all visits appeared inadvertent, with the exception of ants crawling down the corolla to reach nectar. Although there are seven non-native and three native hawkmoth species recorded from Kauaʻi (Nishida, 2002), one native species is likely no longer extant on Kauaʻi (*Manduca blackburni*; USFWS, 2009) and one has not been seen since the summer of 2000 (*Tinostoma smaragditis*; J. Pali, State of Hawaiʻi, Department of Land and Natural Resources, personal communication); none visited the flowers at our ex situ site. Gemmill et al. (1998) recommended coordinated hand pollination between individuals of *B. insignis* to maximize the genetic variability that exists in known accessions. Results of our floral visitor study, which suggest a dramatic reduction or complete loss of pollinators for *B. insignis*, further support this recommendation.

Our pollinator observation study had several limitations. First, the study was only conducted at one site, and in a landscaped garden as opposed to native habitat, because no other large restoration plantings of *B. insignis* occur on Kauaʻi. Allee effects may, therefore, have contributed to the absence of viable pollinators observed in our study, as has been suggested for other moth pollination studies in Hawaiʻi (Weisenberger et al., 2014). Second, nocturnal observations were relatively short (21.5 h) and may not have provided sufficient time to observe moth visitors due to the infrequency of moth visitation to flowers, a trend observed in moth pollinated plants on other oceanic islands (Watanabe et al., 2018). Third, because of time limitations, the fate of the flowers and fruit were not recorded. However, if we infer from the results of our breeding system study, the absence of viable insect pollinators suggests that the plants in our pollinator observation study would likely have been pollen limited with low to no fruit or seed set.

5. Conclusions

Overall, this study highlights the challenges that can face plant

species with specialized pollination mutualisms that now occur in fragmented ecosystems. Due to the combined impacts of invasive species, habitat fragmentation and pollinator loss, *B. insignis* now depends on intense human management to prevent its extinction. The USFWS objectives to prevent extinction of *B. insignis* include restoration of at least three naturally reproducing populations on Kaua'i, with a minimum of 100 mature individuals per population (USFWS, 2017). Our results suggest that without insect assisted outcrossing, *B. insignis* fruit and seed production, and subsequent seedling recruitment, would likely be low in outplanted populations. Since *B. insignis* is cultivated in at least 57 botanical collections around the world, conservation practitioners can work together to identify appropriate pollen donors for inter-institutional crosses and plan for how seed produced should be distributed to improve the genetic and demographic prospects of this species (Fant et al., 2016). However, unless all threats are managed in situ and specialized pollinators are sufficiently abundant to outcross this species in natural settings, the fate of *B. insignis* may forever remain in the hands of ex situ conservationists.

Funding

Financial support was provided to SKW by The Garden Club of America, Hawai'i Community Foundation, Graduate Women in Science, and The Mohamed bin Zayed Species Conservation Fund (Project 13255855). RJP acknowledges the financial assistance provided by Fulbright New Zealand and the John R. Templin Scholarship. RRJ received funding from Deutsche Forschungsgemeinschaft (DFG, JU 2856/2-2).

Data accessibility statement

Readers can locate archived data associated with the listed accession numbers in Appendices A and B at http://lawai.ntbg.org:7324/conservation/.

Declaration of Competing Interest

None.

Acknowledgements

The authors thank NTBG staff who assisted with this project, particularly Mike DeMotta, Steve Perlman, Nicole Shores, Britany Sung, Ashly Trask, and Ken Wood. Alexis Cicciù, Mark Fukada, Will Haines, Karl Magnacca, Aaron Miyamoto, and Steve Montgomery assisted with insect identification. Aaron Shiels, USDA APHIS Wildlife Services, provided advice regarding the statistical analysis; Robert Paull, Department of Tropical Plant and Soil Sciences UHM, undertook the HPLC analyses; and Dan Rubinoff, Department of Plant and Environmental Protection Sciences UHM, provided plants for the study.

Appendix A. Number of *Brighamia insignis* flowers and plants used in the control and each manipulative pollination treatment in each year and in all years combined

Treatment	# of flowers (plants) used in 2011	# of flowers (plants) used in 2012	# of flowers (plants) used in 2013	Total # of flowers (plants) used in study
Control	23 (8)	21 (6)	51 (20)	95 (34)
Self	21 (7)	37 (9)	57 (23)	115 (39)
Self plus	8 (4)	9 (3)	3 (2)	20 (9)
Outcross	18 (6)	3 (3)	18 (13)	39 (22)

Appendix B. Percent pollen viability of all NTBG *B. insignis* accessions and plants examined

NTBG accession #	Plant #	# viable pollen grains	# non-viable pollen grains	Percent viable
120043	3	9	347	3.0%
120043	11	0	300	0.0%
120043	12	0	300	0.0%
990833	4	0	300	0.0%
990833	6	0	300	0.0%
9900842	3	0	300	0.0%
9900842	4	1	299	0.3%
050389	8	0	300	0.0%
050389	8	0	300	0.0%
050389	8	22	278	7.3%
050389	8	78	222	26.0%
050389	8	19	281	6.3%
100651	93	0	300	0.0%
100651	164	1	299	0.3%
100652	1	0	300	0.0%
100652	1	0	300	0.0%
100652	65	0	300	0.0%
990836	2	2	298	0.7%
990836	4	0	300	0.0%
990836	4	0	300	0.0%
990840	1	0	300	0.0%
990840	1	0	300	0.0%
990840	2	223	77	74.3%
990840	3	0	300	0.0%
no accession # on tag	1	0	300	0.0%

Appendix C. List of all NTBG accessions and plants of *B.insignis* used to test pollen viability. Eighteen different individual plants representing nine NTBG accessions were used and 24 replicates total counted to examine pollen viability of plants used in manipulative pollination treatments in 2013

NTBG accession #	Plant # (replicates)
100652	1 (2), 65 (1)
9900842	3 (1), 4 (1)
990836	4 (2), 2 (1)
120043	11 (1), 3 (1), 12 (1)
050389	8 (5)
990840	3 (1), 2 (1), 1 (2)
100651	93 (1), 164 (1)
no accession # on tag	1 (1)
990833	6 (1), 4 (1)

References

Alexander, M.P., 1969. Differential staining of aborted and nonaborted pollen. Stain Technol. 44, 117–122.

Anderson, S.H., Kelly, D., Ladley, J.J., Molloy, S., Terry, J., 2011. Cascading effects of bird functional extinction reduce pollination and plant density. Science 331, 1068–1071.

Aslan, C.E., Liang, C.T., Shiels, A.B., Haines, W., 2018. Absence of native flower visitors for the endangered Hawaiian mint *Stenogyne angustifolia*: impending ecological extinction? Global Ecol. Conserv. 16, e00468.

Aslan, C.E., Shiels, A.B., Haines, W., Liang, C.T., 2019. Non-native insects dominate daytime pollination in a high-elevation Hawaiian dryland ecosystem. Am. J. Bot. 106, 313–324.

Aslan, C.E., Zavaleta, E.S., Croll, D., Tershy, B., 2012. Effects of native and non-native vertebrate mutualists on plants. Conserv. Biol. 26, 778–789.

Aslan, C.E., Zavaleta, E.S., Tershy, B., Croll, D., 2013. Mutualism disruption threatens global plant biodiversity: a systematic review. PLoS One 8, e66993.

Aslan, C.E., Zavaleta, E.S., Tershy, B., Croll, D., Robichaux, R.H., 2014. Imperfect replacement of native species by non-native species as pollinators of endemic Hawaiian plants. Conserv. Biol. 28, 478–488.

Astiz, V., Hernandez, L.F., 2013. Pollen production in sunflower (*Helianthus annuus* L.) is affected by air temperature and relative humidity during early reproductive growth. Int. J. Exp. Bot. 82, 297–302.

Baker, H.G., Baker, I., 1983. Floral nectar sugar constituents in relation to pollinator type. In: Jones, C.E., Little, R.J. (Eds.), Handbook of Experimental Pollination Biology. Scientific and Academic Editions, New York, pp. 117–141.

Banko, W.E., Banko, P.C., 2009. Historic decline and extinction. In: Pratt, T.K., Atkinson, C.T., Banko, P.C., Jacobi, J.D., Woodworth, B.L. (Eds.), Conservation Biology of Hawaiian Forest Birds: Implications for Island Avifauna. Yale University Press, New Haven and London, pp. 25–58.

Bastida, J.M., Alcántara, J.M., Rey, P.J., Vargas, P., Herrera, C.M., 2010. Extended phylogeny of *Aquilegia*: the biogeographical and ecological patterns of two simultaneous but contrasting radiations. Plant Syst. Evol. 284, 171–185.

Bond, W.J., 1994. Do mutualisms matter? Assessing the impact of pollinator and disperser disruption on plant extinction. Philos. Trans. Biol. Sci. 344, 83–90.

Campbell, D.R., 2008. Pollinator shifts and the origin and loss of plant species. Ann. Mo. Bot. Gard. 95, 264–274.

Carlson, J.E., Harms, K.E., 2006. The evolution of gender-biased nectar production in hermaphroditic plants. Bot. Rev. 72, 179–205.

Carpenter, F.L., 1976. Plant-pollinator interactions in Hawai'i: pollination genetics of *Metrosideros collina* (Myrtaceae). Ecology 57, 1125–1144.

Cerino, M.C., Richard, G.A., Torretta, J.P., Gutierrez, H.F., Pensiero, J.F., 2015. Reproductive biology of *Ziziphus mistol* Griseb. (Rhamnaceae), a wild fruit tree of saline environments. Flora 211, 18–25.

Charlesworth, D., Charlesworth, B., 1987. Inbreeding depression and its evolutionary consequences. Annu. Rev. Ecol. Syst. 18, 237–268.

Cory, C., Pender, R., Jones, C.E., 2015. Can ornithophilous Hawaiian lobeliads produce seeds in the absence of pollinators? A test using *Clermontia kakeana* and *Cyanea angustifolia* (Campanulaceae). Pac. Sci. 69, 255–261.

Cox, P.A., Elmqvist, T., 2000. Pollinator extinction in the Pacific Islands. Conserv. Biol. 14, 1237–1239.

Dobson, H.E.M., 2006. Relationship between floral fragrance composition and type of pollinator. In: Dudareva, N., Pichersky, E. (Eds.), Biology of Floral Scent. CRC Press/Taylor & Francis Group, Boca Raton, pp. 147–198.

Donders, T.H., Hagemans, K., Dekker, S.C., de Weger, L.A., de Klerk, P., Wagner-Cremer, F., 2014. Region-specific sensitivity of anemophilous pollen deposition to temperature and precipitation. PLoS ONE 9, e104774.

Dötterl, S., Wolfe, L.M., Jürgens, A., 2005. Qualitative and quantitative analyses of flower scent in *Silene latifolia*. Phytochemistry 66, 203–213.

Elmore, M., 2008. Pollination biology of Hawaiian *Scaevola*. Ms Thesis. University of Hawai'i at Mānoa, Hawai'i, USA.

Fant, J.B., Havens, K., Kramer, A.T., Walsh, S.K., Callicrate, T., Lacy, R.C., Maunder, M., Hird Meyer, A., Smith, P.P., 2016. What to do when we can't bank on seeds: what botanic gardens can learn from the zoo community about conserving plants in living collections. Am. J. Bot. 103, 1541–1543.

Fenster, C.B., Armbruster, W.S., Wilson, P., Dudash, M.R., Thomson, J.D., 2004. Pollination syndromes and floral specialization. Annu. Rev. Ecol. Evol. Syst. 35, 375–403.

Flores-Rentería, L., Whipple, A.V., Benally, G.J., Patterson, A., Canyon, B., Gehring, C.A., 2018. Higher temperature at lower elevation sites fails to promote acclimation or adaptation to heat stress during pollen germination. Front. Plant Sci. 9, 1–14.

Fox, C.W., Reed, D.H., 2010. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. Evolution 65, 246–258.

Gao, J., Xiong, Y.-Z., Huang, S.-Q., 2015. Effects of floral sexual investment and dichogamy on floral longevity. J. Plant Ecol. 8, 116–121.

Gardener, M.C., Daehler, C.C., 2006. Documenting floral visitors to rare Hawaiian plants using automated video recordings. Pac. Conserv. Biol. 12, 189–194.

Gargano, D., Gullo, T., Bernardo, L., 2009. Do inefficient selfing and inbreeding depression challenge the persistence of the rare *Dianthus guliae* Janka (Caryophyllaceae)? Influence of reproductive traits on a plant's proneness to extinction. Plant Species Biol. 24, 69–76.

Gemmill, C.E.C., Ranker, T.A., Ragone, D., Perlman, S.P., Wood, K.R., 1998. Conservation genetics of the endangered endemic Hawaiian genus *Brighamia* (Campanulaceae). Am. J. Bot. 85, 528–539.

Gopalakrishnan, K.K., Thomas, T.D., 2014. Reproductive biology of *Pittosporum dasycaulon* Miq., (Family Pittosporaceae) a rare medicinal tree endemic to Western Ghats. Bot. Stud. 55, 1–11.

Gori, D., 1983. Post-pollination phenomena and adaptive floral changes. In: Little, J. (Ed.), Handbook of Experimental Pollination Biology. Van Nostrand Reinhold Company Inc., New York, pp. 31–49.

Gorresen, P.M., Camp, R.J., Reynolds, M.H., Woodworth, B.L., Pratt, T.K., 2009. Status and trends of native Hawaiian songbirds. In: Pratt, T.K., Atkinson, C.T., Banko, P.C., Jacobi, J.D., Woodworth, B.L. (Eds.), Conservation Biology of Hawaiian Forest Birds: Implications for Island Avifauna. Yale University Press, New Haven, pp. 108–136.

Giambelluca, T.W., Chen, Q., Frazier, A.G., Price, J.P., Chen, Y., Chu, P., Eischeid, J.K., Delparte, D.M., 2013. Online rainfall atlas of Hawai'i. B. Am. Meteorol. Soc. 94, 313–316.

Givnish, T.J., Millam, K.C., Mast, A.R., Paterson, T.B., Theim, T.J., Hipp, A.L., Henss, J.M., Smith, J.F., Wood, K.R., Sytsma, K.J., 2009. Origin, adaptive radiation and diversification of the Hawaiian lobeliads (Asterales: Campanulaceae). Proc. Biol. Sci. 276, 407–416.

Good-Avila, S.V., Nagel, T., Vogler, D.W., Stephenson, A.G., 2003. Effects of inbreeding on male function and self-fertility in the partially self-incompatible herb *Campanula rapunculoides* (Campanulaceae). Am. J. Bot. 90, 1736–1745.

Hannon, D.P., Perlman, S., 2002. The genus *Brighamia*. Cact. Succ. J. 74, 67–76.

Hayes, C.N., Winsor, J.A., Stephenson, A.G., 2005. Multigenerational effects of inbreeding in *Cucurbita pepo* ssp. *texana* (Cucurbitaceae). Evolution 59, 276–286.

Hingston, A.B., McQuillan, P.B., 2000. Are pollination syndromes useful predictors of floral visitors in Tasmania? Austral Ecol. 25, 600–609.

Hoballah, M.E., Stuurman, J., Turlings, T.C.J., Guerin, P.M., Connetable, S., Kuhlemeier, C., 2005. The composition and timing of flower odour emission by wild *Petunia axillaris* coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*. Planta 222, 141–150.

Huppman, E.R.H., 2013. Analysis of Relationships among Endemic Hawaiian *Hibiscus*. Phd Dissertation. University of Hawai'i at Mānoa, Hawai'i, USA.

Johnson, A.L., Ashman, T.-L., 2019. Consequences of invasion for pollen transfer and pollination revealed in a tropical island ecosystem. New Phytol. 221, 142–154.

Johnson, S.D., Moré, M., Amorim, F.W., Haber, W.A., Frankie, G.W., Stanley, D.A., Cocucci, A.A., Raguso, R.A., 2017. The long and the short of it: a global analysis of hawkmoth pollination niches and interaction networks. Funct. Ecol. 31, 101–115.

Johnson, S.D., Neal, P.R., Peter, C.I., Edwards, T.J., 2004. Fruiting failure and limited recruitment in remnant populations of the hawkmoth-pollinated tree *Oxyanthus pyriformis* subsp. *pyriformis* (Rubiaceae). Biol. Conserv. 120, 31–39.

Junker, R.R., Bleil, R., Daehler, C.C., Bluthgen, N., 2010. Intra-floral resource partitioning between endemic and invasive flower visitors: consequences for pollinator effectiveness. Ecol. Entomol. 35, 760–767.

Junker, R.R., Larue-Kontić, A.-A.C., 2018. Elevation predicts the functional composition of alpine plant communities based on vegetative traits, but not based on floral traits.

Alp. Bot. 128, 13–22.
Jürgens, A., Witt, T., Gottsberger, G., 2002. Flower scent composition in night-flowering *Silene* species (Caryophyllaceae). Biochem. Syst. Ecol. 30, 383–397.
Kaiser, R., 2010. Scent of the Vanishing Flora. Verlag Helvetica Chimica Acta, Zurich.
Knudsen, J.T., Eriksson, R., Gershenzon, J., Stahl, B., 2006. Diversity and distribution of floral scent. Bot. Rev. 72, 1–120.
Koch, J.B., Sahli, H.F., 2013. Patterns of flower visitation across elevation and successional gradients in Hawai'i. Pac. Sci. 67, 253–266.
Krushelnycky, P.D., 2014. Evaluating the interacting influences of pollination, seed predation, invasive species and isolation on reproductive success in a threatened alpine species. PLoS One 9, e88948.
Kuppler, J., Höfers, M.K., Trutschnis, W., Bathke, A.C., Eiben, J.A., Daehler, C.C., Junker, R.R., 2017. Exotic flower visitors exploit large floral trait spaces resulting in asymmetric resource partitioning with native visitors. Functi. Ecol. 31, 2244–2254.
Lagomarsino, L.P., Condamine, F.L., Antonelli, A., Mulch, A., Davis, C.C., 2016. The abiotic and biotic drivers of rapid diversification in Andean bellflowers (Campanulaceae). New Phytol. 210, 1430–1442.
Lammers, T.G., Weller, S.G., Sakai, A.K., 1987. Japanese white-eye, an introduced passerine, visits the flowers of *Clermontia arborescens*, an endemic Hawaiian lobelioid. Pac. Sci. 41, 74–78.
Lammers, T.G., 1989. Revision of *Brighamia* (Campanulaceae: Lobelioideae) a caudiciform succulent endemic to the Hawaiian Islands. Syst. Bot. 14, 133–138.
Lammers, T.G., 1999. Campanulaceae. In: Wagner, W.L., Herbst, D.R., Sohmer, S.H. (Eds.), Manual of the Flowering Plants of Hawai'i. University of Hawai'i Press, Honolulu, pp. 420–489.
Lammers, T.G., Freeman, C.E., 1986. Ornithophily among the Hawaiian Lobelioideae (Campanulaceae): evidence from floral nectar sugar compositions. Am. J. Bot. 73, 1613–1619.
Langenberger, M.W., Davis, A.R., 2002. Temporal changes in floral nectar production, reabsorption, and composition associated with dichogamy in annual caraway (*Carum carvi*; Apiaceae). Am. J. Bot. 89, 1588–1598.
Lau, T.-C., Stephenson, A.G., 1993. Effects of soil nitrogen on pollen production, pollen grain size, and pollen performance in *Cucurbita pepo* (Cucurbitaceae). Am. J. Bot. 80, 763–768.
Martinell, M.C., Dötterl, S., Blanché, C., Rovira, A., Massó, S., Bosch, M., 2010. Nocturnal pollination of the endemic *Silene sennenii* (Caryophyllaceae): an endangered mutualism? Plant Ecol. 211, 203–218.
Martins, D.J., Johnson, S.D., 2007. Hawkmoth pollination of aerangoid orchids in Kenya, with special reference to nectar sugar concentration gradients in the floral spurs. Am. J. Bot. 94, 650–659.
Martins, D.J., Johnson, S.D., 2013. Interactions between hawkmoths and flowering plants in East Africa: polyphagy and evolutionary specialization in an ecological context. Biol. J. Linn. Soc. Lond. 110, 199–213.
Medeiros, M.J., Eiben, J.A., Haines, W.P., Kaholoaa, R.L., King, C.B.A., Krushelnycky, P.D., Magnacca, K.N., Rubinoff, D., Starr, F., Starr, K., 2013. The importance of insect monitoring to conservation actions in Hawai'i. Proc. Hawaii. Entomol. Soc. 45, 149–166.
Mercuri, A.M., Torri, P., Casini, E., Olmi, L., 2013. Climate warming and the decline of *Taxus* airborne pollen in urban pollen rain (Emilia Romagna, northern Italy). Plant Biol. 15, 70–82.
Mitchell, T.C., Dötterl, S., Schaefer, H., 2015. Hawk-moth pollination and elaborate petals in Cucurbitaceae: the case of the Caribbean endemic *Linnaeosicyos amara*. Flora 216, 50–56.
Nishida, G.M., 2002. Hawaiian Terrestrial Arthropod Checklist. Bishop Museum Technical Report No. 22. Hawai'i Biological Survey, Bishop Museum, Honolulu.
Norman, J.K., Weller, S.G., Sakai, A.K., 1997. Pollination biology and outcrossing rates in hermaphroditic *Schiedea lydgatei* (Caryophyllaceae). Am. J. Bot. 84, 641–648.
NTBG, 2018. Collections Management Database System. Retrieved online August 27, 2018. http://lawai.ntbg.org:7324/.
Ollerton, J., Alarcón, R., Waser, N.M., Price, M.V., Watts, S., Cranmer, L., Hingston, A., Peter, C.I., Rotenberry, J., 2009. A global test of the pollination syndrome hypothesis. Ann. Bot. 103, 1471–1480.
Paulino-Neto, H.F., 2013. Floral biology and breeding system of *Bauhinia forficata* (Leguminosae: Caesalpinioideae), a moth-pollinated tree in southeastern Brazil. Braz. J. Bot. 36, 55–64.
Pender, R.J., Morden, C.W., Paull, R.E., 2014. Investigating the pollination syndrome of the Hawaiian lobeliad genus *Clermontia* (Campanulaceae) using floral nectar traits. Am. J. Bot. 101, 201–205.
Peter, C.I., Coombs, G., Huchzermeyer, C.F., Venter, N., Winkler, A.C., Hutton, D., Papier, L.A., Dold, A.P., Johnson, S.D., 2009. Confirmation of hawkmoth pollination in *Habenaria epipactidea*: leg placement of pollinaria and crepuscular scent emission. S. Afr. J. Bot. 75, 744–750.
Peterson, R., Slovin, J.P., Chen, C., 2010. A simplified method for differential staining of aborted and non-aborted pollen grains. Int. J. Plant Biol. 1, 66–69.
Pleasants, J.M., Wendel, J.F., 2010. Reproductive and pollination biology of the endemic Hawaiian cotton, *Gossypium tomentosum* (Malvaceae). Pac. Sci. 64, 45–55.
Raguso, R.A., Levin, R.A., Foose, S.E., Holmberg, M.W., McDade, L.A., 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in Nicotiana. Phytochemistry 63, 265–284.
Riffell, J.A., Lei, H., Abrell, L., Hildebrand, J.G., 2012. Neural basis of a pollinator's buffet: olfactory specialization and learning in *Manduca sexta*. Science 339, 200–204.
Riffell, J.A., Lei, H., Hillebrand, J.G., 2009. Neural correlates of behavior in the moth *Manduca sexta* in response to complex odors. Proc. Natl. Acad. Sci. U. S. A. 106, 19219–19226.
Rodger, J.G., Ellis, A.G., 2016. Distinct effects of pollinator dependence and self-incompatibility on pollen limitation in South African biodiversity hotspots. Biol. Lett. 12, 20160253.
Sahli, H.F., Krushelnycky, P.D., Drake, D.R., Taylor, A.D., 2016. Patterns of floral visitation to native Hawaiian plants in presence and absence of invasive Argentine ants. Pac. Sci. 70, 309–322.
Sakai, A.K., Wagner, W.L., Mehrhoff, L.A., 2002. Patterns of endangerment in the Hawaiian flora. Syst. Biol. 51, 276–302.
Schemske, D.W., Lande, R., 1985. The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. Evolution 39, 41–52.
Shay, K., Drake, D.R., Taylor, A.D., Sahli, H.F., Euaparadorn, M., Akamine, M., Imamura, J., Powless, D., Aldrich, P., 2016. Alien insects dominate the plant-pollinator network of a Hawaiian coastal ecosystem. Pac. Sci. 70, 409–429.
Stebbins, G.L., 1957. Self fertilization and population variability in the higher plants. Am. Nat. 91, 337–354.
Stratton, D.A., 1989. Longevity of individual flowers in a Costa Rican cloud forest: ecological correlates and phylogenetic constraints. Biotropica 21, 308–318.
Tangmitcharoen, S., Owens, J.N., 1997. Pollen viability and pollen-tube growth following controlled pollination and their relation to low fruit production in teak (*Tectona grandis* Linn. f.). Ann. Bot. 80, 401–410.
USFWS, 2007. *Brighamia insignis* (Olulu) 5-Year Review: Summary and Evaluation. U.S. Fish and Wildlife Service, Pacific Island Fish and Wildlife Office, Honolulu, Hawai'i.
USFWS, 2009. Blackburn's Sphinx Moth (*Manduca blackburni*) 5-Year Review: Summary and Evaluation. U.S. Fish and Wildlife Service, Pacific Island Fish and Wildlife Office, Honolulu, Hawai'i.
USFWS, 2017. 5-Year Review Short Form Summary: *Brighamia insignis* (Ōlulu). U.S. Fish and Wildlife Service, Pacific Island Fish and Wildlife Office, Honolulu, Hawai'i.
USFWS, 2018. U.S. Fish and Wildlife Service's Endangered Species Database. Retrieved online April 18, 2018. http://www.fws.gov/endangered/.
van der Niet, T., Jürgens, A., Johnson, D., 2015. Is the timing of scent emission correlated with insect visitor activity and pollination in long-spurred *Satyrium* species? Plant Biol. 17, 226–237.
Wagner, W.L., Herbst, D.R., Sohmer, S.H., 1999. Manual of the Flowering Plants of Hawai'i. University of Hawai'i Press, Honolulu.
Wagner, W.L., Khan, N.R., Lorence, D.H., Herbst, D.R., 2014. Native vascular plants of the Hawaiian Islands. In: Gustafson, R.J., Herbst, D.R., Rundel, P.W. (Eds.), Hawaiian Plant Life: Vegetation and Flora. University of Hawai'i Press, Honolulu, pp. 255–287.
Walsh, S., 2016. *Brighamia insignis*. The IUCN Red List of Threatened Species 2016. e.T44080A83789215. Downloaded October 7, 2016. .
Watanabe, K., Kato, H., Kuraya, E., Sugawara, T., 2018. Pollination and reproduction of *Psychotria homalosperma*, an endangered distylous tree endemic to the oceanic Bonin (Ogasawara) Islands, Japan. Plant Spec. Biol. 33, 16–27.
Weisenberger, L., Keir, M.J., 2014. Assessing status, capacity, and needs for the ex situ conservation of the Hawaiian flora. Pac. Sci. 68, 525–536.
Weisenberger, L.A., Weller, S.G., Sakai, A.K., 2014. Remnants of populations provide effective source material for reintroduction of an endangered Hawaiian plant, *Schiedea kaalae* (Caryophyllaceae). Am. J. Bot. 101, 1954–1962.
Weller, S.G., Sakai, A.K., Campbell, D.R., Powers, J.M., Pena, S.R., Keir, M.J., Loomis, A.K., Heintzman, S.M., Weisenberger, L., 2017. An enigmatic Hawaiian moth is a missing link in the adaptive radiation of *Schiedea*. New Phytol. 213, 1533–1542.
Whittall, J.B., Hodges, S.A., 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447, 706–709.
Wilcock, C., Neiland, R., 2002. Pollination failure in plants: why it happens and when it matters. Trends Plant Sci. 7, 270–277.
Wolfe, A.D., McMullen-Sibul, A., Tepedino, V.J., Kubatko, L., Necamp, T., Fassnacht, S., 2014. Conservation genetics and breeding system of *Penstemon debilis* (Plantaginaceae), a rare beardtongue endemic to oil shale talus in western Colorado, USA. J. Syst. Evol. 52, 598–611.
Wolff, D., 2006. Nectar sugar composition and volumes of 47 species of Gentianales from a Southern Ecuadorian montane forest. Ann. Bot. 97, 767–777.
Wood, K.R., Appelhans, M.S., Wagner, W.L., 2016. *Melicope oppenheimeri*, section *Pelea* (Rutaceae), a new species from West Maui, Hawaiian Islands: with notes on its ecology, conservation, and phylogenetic placement. PhytoKeys 69, 51–64.
Zimmerman, E.C., 1948. Insects of Hawai'i, vol. 1 Introduction. University of Hawai'i Press, Honolulu.
Zimmerman, E.C., 1958. Insects of Hawai'i, vol. 7 Macrolepidoptera. University of Hawai'i Press, Honolulu.
Zimmerman, E.C., 1978. Insects of Hawai'i, vol. 9 Microlepidoptera. University of Hawai'i Press, Honolulu.