Pollen on Stigmas of Herbarium Specimens: A Window into the Impacts of a Century of Environmental Disturbance on Pollen Transfer

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abstract: Pollination is necessary for plant reproduction but often highly susceptible to disruption, for example, by habitat fragmentation and climate change. Here, we indirectly evaluated on a century timescale pollination interactions for species in one of the historically most disturbed habitats on earth—tropical dry forests of Hawai‘i. We employed a novel method for acquiring a historical perspective on temporal change in pollination by characterizing pollen on stigmas of herbarium specimens from six remnant native species collected from 1909–2002. We determine whether temporal shifts occurred in (1) pollination quantity and quality or (2) the composition of species interacting via pollen transfer. While pollen quantity remained constant, these remnant species interact with different species in modern times via pollen transfer than they did nearly 100 years ago. Species that are resilient to long-term environmental change may also be the ones subject to changes in pollination interactions.

Keywords: disturbance, Hawai‘i, herbarium, interspecific pollen transfer, pollination, tropical dry forest.

Introduction

Mutualisms between animals and flowering plants are critical to the functioning of terrestrial ecosystems around the world and are also increasingly threatened as a result of anthropogenic disturbances (Kearns et al. 1998; Potts et al. 2010). These interactions have become a focus of applied conservation research as a metric for characterizing how shifts in species diversity translate into shifts in ecological functioning within communities. For example, measuring how pollination interactions change along disturbance gradients (e.g., Burké and Alarcon 2011; Johnson and Ashman 2019) or after restoration management activities have occurred (e.g., Fontúrbel et al. 2017; Kaiser-Bunbury et al. 2017) can provide valuable information about the functional impacts of species gains and losses in threatened ecosystems.

It has proven difficult to use pollinator or plant traits to make general predictions about the effects of shifts in plant-pollinator outcomes (Bartomeus et al. 2018). This suggests that we need to directly quantify the complex relationships between shifting plant and pollinator communities and pollination services. For example, while empirical studies of pollination interactions over multiple seasons show substantial temporal variation and the ability to rewire on short timescales to adjust to the loss and gain of individual species (Olesen et al. 2008; CaraDonna et al. 2017), we have far less of an understanding of the impact of these changes over the longer ecological time frames over which true extinctions and invasions occur.

An additional challenge is that often observational measures of pollinator visits to flowers do not directly correlate with the deposition of pollen grains on floral stigmas (e.g., King et al. 2013). Pollinators vary in their effectiveness (Waser et al. 1996), leading to indirect correspondence between the numbers of visits observed and the actual quantity of conspecific pollen (a measure of pollination quantity) as well as heterospecific pollen (a component of pollination quality) deposited on plant stigmas (Engel and Irwin 2003; Alarcón 2010). Thus, direct measures of pollen grain identity and quantity deposited on plant stigmas can represent an integrated measure of the outcome of all pollinator visits over the lifetime of the individual flower (Ashman and Arceo-Gomez 2013). Focusing on heterospecific pollen deposition patterns that can have a variety of negative consequences...
for plant reproduction (Ashman and Arceo-Gomez 2013) can also provide a record of plants that both share pollinators and interact directly via pollen transfer (Fang and Huang can also provide a record of plants that both share pollinators for plant reproduction (Ashman and Arceo-Gomez 2013).

Here we explore how pollination interactions, as measured by pollen received by stigmas, have shifted over more than a century of dramatic environmental change in the dry tropical forest ecosystems of the island of Hawai‘i, Hawaii, one of the most threatened ecosystem types in the world (Hoekstra et al. 2004; Miles et al. 2006). To examine how disturbances leading to shifting species composition over the past century (fig. 1) have altered patterns of pollination interactions, we employed a novel method for tracking temporal change in pollination by recording pollen received by the stigmas of herbarium specimens from six remnant native dry forest species collected on the big island of Hawai‘i from 1909–2002. Additionally, for qualitative comparison we returned to remnant dry forest sites in the regions where herbarium specimens were collected and collected contemporary samples from the same species. We ask, first, whether there were shifts in pollination quantity and quality over time, as measured by the amount of conspecific pollen received as well as the proportion and diversity of heterospecific pollen found on stigmas, and, second, whether the composition of species interacting via pollen transfer changed substantially over time.

Methods
Study System
The flora of the Hawaiian Islands is considered one of the most vulnerable insular floras in the world to invasive species as well as to habitat loss and climate change (Caujapé-Castells et al. 2010). The largest remnant patches of dry tropical forest in the Hawaiian islands today are located on Hawai‘i island, where we focused our study (fig. 1), and after many years of intense disturbance are increasingly the focus of conservation and restoration efforts (e.g., Cabin et al. 2000; Friday et al. 2015).

The Hawaiian forests were the site of great exploration and botanical activity in the early 1900s, with prominent naturalists such as Joseph Rock, Otto Degener, and Charles Noyes Forbes providing large numbers of specimens to museum collections around the world. The collection of botanical specimens began while much of the upland dry forest ecosystems remained relatively undisturbed and continued throughout a century of dramatic environmental change across the Hawaiian Islands (fig. 1). We focus on six native dry forest plant species from six families with a range of floral morphology (table S1; tables S1–S3 are available online) and for which we have evidence of variation in the mean proportion of heterospecific pollen they receive (Johnson and Ashman 2019). These also all remain relatively common there to-day. Specimens used for this study were made available from the Herbarium Pacificum (BISH) collection of the Hawaiian flora; they were selected for inclusion on the basis of geographic proximity to contemporary remnant dry forest patches and for availability within the herbarium specimen sheet of accessible stigmas from flowers at the late stage of anthesis to ensure that pollen on stigmas integrated interactions across the full lifetime of the flower. Herbarium sample locations were determined on the basis of qualitative notes provided on herbarium sheets by collectors (fig. 1). We quantitatively test hypotheses for pollination change within the 100-year span of herbarium specimens but acknowledge that an untested assumption of these analyses is that the handling, pressing, drying, mounting, and long-term storage of herbarium specimens affects pollen grain retention on stigmas similarly across the range of specimen ages. To provide a benchmark as to how the historical pollination metrics compare with a contemporary data set, we qualitatively contrast the historical records to field collections of stigmas from the same remnant dry forest species from the same collection regions in the spring of 2016 (fig. 1) and reported by Johnson and Ashman (2019).

Data Collection
Stigmas were collected from the following species: hopbush, Dodonaea viscosa (DOVI), Sapindaceae; ʻōhi‘a lehua, Metrosideros polymorpha (MEPO), Myrtaceae; false sandalwood, Myoporum sandwicense (MYSA), Scrophulariaceae; Hawaiian hawthorn, Osteomeles anthyllidifolia (OSAN), Rosaceae; mamāne, Sophora chrysophylla (SOPH), Fabaceae; and ʻōhelo ʻai, Vaccinium reticulatum (VARE), Ericaceae. All species are biotically pollinated, including DOVI, which is insect and wind pollinated (P. Aldrich, unpublished data; A. L. Johnson and T.-L. Ashman, personal observation), suggesting that transport of heterospecific pollen to stigmas is pollinator mediated. In all, we collected data on stigmatic pollen loads from 145 herbarium specimens (totals for pre-1950 [53] and post-1950 [92] time periods for each species; DOVI: 6, 12; MEPO: 4, 15; MYSA: 21, 33; OSAN: 5, 3; SOPH: 8, 14; VARE: 8, 16) by excising a single stigma per herbarium sheet into centrifuge tubes containing 70% ethanol. Stigmas were then acetylated (Kearns and Inouye 1993), and pollen samples were mounted on slides where grains were counted and identified to species at ×400 magnification using a compound light microscope (Olympus, CX41). Pollen grains that we were unable to identify to species on the basis of contemporary reference collections (Johnson and Ashman 2019) were grouped into categories by size, shape, and texture and treated as individual pollen morphospecies in subsequent analyses. Further identification of these was conducted when possible on the basis of images from Selling (1947). For qualitative comparisons to contemporary data
**Figure 1:** Historical and geographical context in which this study took place. Map depicts georeferenced location of herbarium samples on Hawai‘i in relation to the two remnant forest reserves (stars) where contemporary samples were taken, with time of collection (from oldest to most recent) indicated by color (black = oldest samples; white = most recently collected herbarium samples). Time line describes the environmental history of the dry forest in Hawai‘i, with a focus on disturbances that have impacted both pollinator and floral biodiversity (bottom of time line) and descriptions of the state of dry forest communities made by prominent regional botanists (top of time line). For relevant historical references used to construct time line, see table S2 (available online).
collected by Johnson and Ashman (2019), 98 samples of the six focal species from that study were evaluated similarly in this study.

**Statistical Analysis**

Herbarium specimens were divided into two broad temporal categories for analysis: pre-1950 (1909–1949) and post-1950 (1950–2002). This division was selected on the basis of the environmental history of the region and the temporal distribution of collections (table S1). Specifically, while in 1913 the dry forest regions of western Hawai‘i were considered relatively diverse and intact, by Joseph Rock’s 1955 account the forests had been significantly impacted. That is, upland forests had been converted to pasture and cropland, and introduced plants were spreading. At the same time, native pollinating bird populations were in decline and honeybees were well established as introduced pollinators (fig. 1). Few samples spanned 1950–1955, so by taking 1950 as the dividing line we maximized the number of samples collected during the time when the forests were intact. No samples collected from the focal sites after 2002 met our requirements for sampling.

We compared (1) conspecific pollen quantity, (2) proportion of total pollen that was heterospecific (hereafter, heterospecific pollen proportion), and (3) heterospecific pollen morphospecies richness of herbarium specimens across the two time periods (pre-1950 and post-1950) using generalized linear mixed models (GLMMs). We included species in each model as a random (blocking) effect. We used zero inflated negative binomial distributions for the models containing count data (number of conspecific pollen grains and heterospecific pollen morphospecies richness) and a tweedie model for the continuous variable (heterospecific pollen proportion). To assess whether including time period in the model improved model fit, we compared the full model, if significant, with a null model containing just the random species effects, using a likelihood ratio test (Zuur et al. 2009). We also examined the shifting composition of interactions via pollen transfer over time, using a subset of four of the six species with the largest sample size (table S1; this analysis included DOVI, MEPO, MYSA, and VARE) and analyzing only stigmas that had received heterospecific pollen. We performed a permutational ANOVA (PERMANOVA), stratified by species, to determine whether the composition of species interacting shifted across the two time periods. We also tested for a shift in compositional variance rather than species identity, using a multivariate homogeneity of group dispersions test (Anderson 2006) followed by a permutational test for significance of the model. We visualized these results using a principle coordinates analysis (PCoA) ordination. To assess the robustness of our conclusions, we evaluated (1) the effect of sample size on the GLMMs informally by simulating increased sample size from the same data distribution and (2) sample size adequacy on composition by comparing our observed data (PCoA) with a simulated distribution of the null expectation of multidimensional Euclidian distances between the centroids of each time point and by employing the method of Anderson and Santana-Garcon (2015) for precision in multivariate assemblage data via PERMANOVA (for details, see figs. A1, A2, available online). All analyses were conducted in R (R Development Core Team 2015). To further visualize shifts in composition across historical time periods and relative to the contemporary data, we also constructed directed network diagrams depicting heterospecific pollen transfer, using the igraph package in R (Csardi and Nepusz 2006); for detailed description of methods for creating directed networks describing heterospecific pollen transfer, see Johnson and Ashman (2019).

**Results**

**Pollination Quality and Quantity**

Conspecific pollen receipt showed no change over time ($Z = -0.005, P = .99$; fig. 2A). Heterospecific pollen proportion, however, increased by 33% between pre-1950 and post-1950 samples (0.13 vs. 0.19; $Z = -2.158, P = .03$; fig. 2B). The maximum likelihood test comparing the full model to the null model, however, suggested that the full model was only a marginally significantly better fit than the null model (Akaike information criterion $= 1, P = .08$). There was no difference across time periods in heterospecific pollen morphospecies richness ($Z = 1.481, P = .14$; fig. 2C). Informal power analyses suggested that sample size did limit the power of the analyses of heterospecific pollen proportion but not the other metrics. The single contemporary time point was qualitatively similar in heterospecific pollen metrics though lower in conspecific pollen loads for mean and SE for each sampling period, see table S3). Data underlying figure 2 and taken at the contemporary sampling period have been deposited in the Dryad Digital Repository (https://dx.doi.org/10.5061/dryad.34n4t78; Johnson et al. 2019).

**Composition of Pollination Interactions**

While we found no substantial difference in compositional variance between time periods ($F = 0.324, P = .95$), compositional identity varied significantly (PERMANOVA $F = 2.055, P = .02$). These results were corroborated by PCoA visualization of compositional relationships across time periods and plant species (fig. 3). Centroids representing pre-1950 heterospecific pollen were distinct in compositional space from post-1950 heterospecific pollen for three out of four species in this analysis (DOVI, MYSA, and VARE; not
Figure 2: Comparison of pollen quantity and quality across sampling time periods. A. Number of conspecific pollen grains per sample. B. Proportion of heterospecific pollen per sample. C. Richness of species donating heterospecific pollen to each stigma sample. Each point represents one sample, color coded by species. Codes are described in table S1 (available online).
MEPO), indicating that composition of the pollen on stigmas changed over time. Analyses of the robustness of these conclusions (figs. A1, A2) indicate that sample sizes were more than enough to distinguish between the two time periods for DOVI, marginally sufficient for MYSA and VARE, but insufficient for MEPO. Likewise, the evaluation of the sample sizes for the PERMANOVA indicated that across all species pooled the sample sizes were sufficient but that individually MEPO and DOVI fell below the indicated eight to 10 stigmas per species in pre-1950. Nonetheless, all species comparisons are qualitatively consistent with the overall pattern of community shifts and significant when power was adequate. Visualization of the network interactions observed across the historical time periods also supported this observation, as networks appeared much less dense and connected in the post-1950 relative to the pre-1950 sampling period (fig. S1, available online). These network changes are also apparent in the contemporary network (fig. S1). Species added to the networks were primarily introduced species, such as Madagascar ragwort (Senecio madagascariensis) and crimson fountaingrass (Pennisetum setaceum). Species that were lost between progressive sampling periods were likely native species, such as the identifiable native species lama (Diospyros sandwicensis), but could be the numerous unidentified pollen morphospecies found only in the pre-1950 sampling period.

**Discussion**

By taking an unprecedented long-range view of pollination interactions, we have demonstrated that substantial changes that have occurred in Hawaiian dry forest cover and composition over the past century have also led to alteration of the composition of the community of plant species that interact via pollination. By tracking pollen transfer for six common native species over a century, we demonstrated that remnant native species today interact with very different plants via pollen transfer than they did 100 years ago. These changes in plant-plant interactions mediated by pollinators could have long-term evolutionary implications, especially if the new community of interacting species vary phenotypically or phylogenetically from the pre-1950 co-flowering community. Shifts in pollinator-mediated plant interactions reflected in the pollen communities on stigmas could exert novel selective pressures on floral traits that mediate pollen transfer (e.g., flowering time, morphology) especially if receipt of novel (invasive) heterospecific pollen has significant negative effects on reproduction (Eckert et al. 2010; Kiers et al. 2010; Beans and Roach 2015; Arceo-Gomez and Ashman 2016).

Our results complement several other empirical studies of pollination interactions, which similarly highlight the plasticity of interactions and the ability for many plant
species to persist in rewired networks (Petanidou et al. 2008; Burkle et al. 2013; Montero-Castaño and Vilà 2017). No significant shifts in the quantity of conspecific pollen received by focal flowering species were observed in the herbarium samples. However, there was evidence of increased heterospecific proportion. The introduced honeybee may be successfully replacing native pollinator losses over the past 50 years—at least for these focal species—in terms of conspecific pollen transfer, but these findings may also indicate that the honeybee is not serving as a fully functional substitution (e.g., Aslan et al. 2012) because its high levels of generalism might be responsible for the higher proportion heterospecific pollen (fig. 2B; also Johnson and Ashman 2019). Increased invasive plant density could also have increased available heterospecific pollen. Qualitatively, the contemporary data set had lower conspecific pollen receipt, but this could be due to a number of factors, including the El Nino drought during sampling, dramatic plant community changes, recent declines in honeybee populations in Hawai‘i (a result of the Varroa mite; Martin et al. 2012), or acceleration of declines in native pollinator populations (Magnacca and King 2013). Further contemporary data sets are needed to determine whether there is persistent drop in pollination services in this system.

Our work additionally demonstrates the value of a novel method of quantifying changes in patterns of pollination interactions—a key measure of ecological functioning of communities—over time. Tracking shifts in pollination based on stigmatic pollen loads of herbarium specimens is a viable approach to quantifying changes over time in pollination interactions via realized pollen transfer and represents an additional valuable and unexpected use for biological collections (Heberling and Isaac 2017). This approach provides a complementary perspective on these changes to that provided by pollen analyzed on insect collections or historical plant-pollinator interaction data sets (Burkle et al. 2013). While one needs to acknowledge sample size limitations imposed by use of rare preserved specimens and assumptions of equal retention of pollen grains on the stigmas of preserved flowers, our work shows that important changes in pollination can be robust to these limitations (fig. A1). For instance, DOVI shows pronounced compositional shifts in pollen received over the span of the study. Interestingly, this species is dioecious and morphologically adapted for wind pollination but is often visited by honeybees (Johnson and Ashman 2019; P. Aldrich, unpublished data; A. L. Johnson and T.-L. Ashman, personal observation), suggesting that increased use of this species by honeybees could have driven this pattern of heterospecific pollen receipt.

Because we focused on species that have remained common and relatively successful in the midst of a rapidly changing ecosystem, our estimate of the changes in pollination quantity and quality that have occurred in these ecosystems over time is likely to be conservative. Plant species that suffered precipitous declines or were driven toward extinction over the past century may have also experienced much more dramatic shifts in pollination quantity and quality than we observed. For example, the Hawaiian flora has a very high rate of doecy (Sakai et al. 1995), and these obligately outcrossing species could be more susceptible to population declines following the disruption of specialist pollination interactions. Species missing from the pollination transfer networks that were present pre-1950 could be indicative of these lost interactions (e.g., Diospyros sandwicensis; fig. S1).

The connections between shifts in community diversity or abundance, patterns of species interactions, and ecological functioning are complex and often indirect (Chapin et al. 1997; Tylianakis et al. 2008; Valiente-Banuet et al. 2015). Studies that, like ours, empirically document temporal shifts in pollination interactions over long periods of time are key to better understanding links between the changing composition of species communities as a result of long-term and broadscale disturbances. This is especially true because shifts in pollinator-mediated plant-plant interactions on the stigma could reduce plant reproduction, as both amount and composition of pollen can affect seed production (Arceo-Gomez and Ashman 2011, 2016). Complementary studies that monitor ecological interactions after restoration actions have been implemented are also necessary for ensuring that management decisions focused on increasing species diversity are also leading to more functional communities (e.g., Kaiser-Bunbury et al. 2017). But we should note that demonstrating fitness impacts of pollinator changes is necessary to validate functional changes. Tracking how shifting interactions may alter selection—for example, by linking changing pollination interactions with changes in floral morphology (e.g., Smith and Kriebel 2017), flowering phenology (e.g., Wolf et al. 2017), or stigma phenotype (Ashman and Arceo-Gomez 2013) over time—may provide valuable insight into how species can avoid or tolerate heterospecific pollen receipt (Ashman and Arceo-Gomez 2013) and thus be resilient to compositional shifts in coflowering communities and mutualistic partners.

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Left, stigmas and anthers with visible pollen on an herbarium specimen of *Metrosideros polymorpha*, viewed through a dissecting scope. Right, *Metrosideros polymorpha* being visited by a honey bee in the field. Photo credit: Anna L. Johnson.