

The role of pollen identification in examining butterfly pollination networks

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ABSTRACT

Pollinator-plant mutualisms have resulted with developed plant characteristics designed to encourage pollinators to provide pollination services. These mutualisms are particularly noteworthy as 87% of all flowering plants and 75% of all crop plants are positively influenced by animal pollination. According to this pollination syndrome model, most pollinators would be considered specialized with respect to their entire floral community, as they most frequently visit flowers with particular characteristics. However, there is increasing evidence indicating that pollinators are more generalized than once believed. Understanding the structure of pollination networks is key to uncovering the true level of specialization within that network; thus I investigated whether the incorporation of pollen analysis can provide a more accurate representation of the total pollinator-plant interactions than visual surveys alone. I analyzed pollen on 75 butterfly specimens collected from five meadows in the Madrean Sky Islands and compared the pollinator-plant interactions recorded in the pollen to the interactions observed in the field. I found that the pollen data provided a record of more pollinator-plant interactions than the observation data. The pollen data revealed that most species were more generalized than they appeared in the observation data, because I was able to identify additional floral interaction partners that visual surveys missed. The incorporation of pollen data created a more complex network structure, and as a result should be taken into consideration when conducting network studies to allow for more clarity on the level of pollinator specialization within the network.

KEYWORDS

Specialization, generalization, modularity, plant-pollinator interactions, pollen analysis, network

INTRODUCTION

Pollinators collect pollen for different reasons— some intentionally gather it for food while others inadvertently accumulate it while landing on flowers for other purposes (Pacini 2008). Butterflies, members of the Lepidoptera order, visit flowers for the purpose of collecting nectar as a food source. Consequently, they are classified as accidental pollinators that unintentionally pick up pollen on their legs and body while perching on flowers to forage for nectar (Boggs and Ross 1993, Inouye 2007).

Many plants and pollinators have co-evolved, and as a result, many flowering plants have developed characteristics such as nectar, colors, and scents that encourage pollinators to visit their flowers. Different combinations of floral characteristics are believed to attract subsets of the pollinator community, which are referred to as pollination syndromes. Although these characteristics are used to predict which flowers pollinators visit, there is evidence that suggests pollinators do not necessarily adhere to this pollination syndrome model (Ollerton et al. 2009).

According to the pollination syndrome model, most pollinators would fall into one of the subsets of floral characteristic combinations and therefore would express higher levels of floral selectivity and specialization (Blüthgen et al. 2006). Pollinators are traditionally classified as either generalists or specialists; generalists visit a wide range of flower types to forage for pollen or nectar, while specialists visit a few or even a single plant species. The wide spectrum of visitation behaviors, however, makes it difficult to classify pollinators based on this dichotomous scale (Johnson & Steiner 2000, Waser & Ollerton 2006). The degree of specialization in plant-pollinator relationships is currently a highly debated topic, and there is increasing skepticism about the widespread specialization previously thought to be present in pollination systems (Waser et al. 1996, Johnson & Steiner 2000). The controversy highlights the need for further experimental investigation of pollination systems (Waser et al. 1996, Johnson and Steiner 2000), which is often conducted through observational studies.

Exploring the structure of pollination networks is key to understanding pollinator-plant relationships and co-evolutionary processes (Bosch et al. 2009). Network analyses often rely on visual surveys focusing on pollinator visitation of each plant species (Bosch et al. 2009). Studies relying solely on observational surveys often find high numbers of specialists, which contradicts the increasingly accepted notion that generalization is the norm within pollination networks

(Vázquez & Aizen 2004, Bosch et al. 2009). Sampling and identifying pollen grains collected from the bodies of pollinators results in increased network connectance (Bosch et al. 2009). The pollen grains pollinators collect from multiple plants will remain on their body for extended periods of time and can therefore provide insights into multiple pollinator-plant interactions (Courtney et al. 1982). However, the power of pollen analysis has not been fully established for all ecosystem types.

To better understand the level of specialization actually present within pollination systems, I investigated the difference between two collection methods and their respective estimation of specialization. Using butterfly specimens collected from five alpine meadows in the Madrean Sky Islands, I will compare the plants each pollinator species was observed visiting to the pollen grain collected off the bodies of the pollinators. My objective is to (1) determine if pollen analysis reveals significantly more pollinator-plant interactions compared to observation data alone. I also will (2) determine if butterflies exhibit specialist or generalist behavior, and (3) observe if this behavior differs between the observation and pollen data. I expect that the addition of pollen data will yield additional network connections not observed with observation data alone. Additionally, I anticipate that butterflies will appear to be specialists when examining the observation data, but will appear to be more generalized with the addition of pollen data.

METHODS

Study site

I studied butterfly specimens collected from higher elevation meadows in the Madrean Sky Islands of New Mexico and Arizona. More than 250 butterfly specimens were collected from the Sangre de Cristo (JC), Sandias (SC), Magdalena (MM), Pinalaño (PL), and Chiricahua (CH) mountains. The specific meadows studied were Johnson's mesa and Jack's Creek Trail meadows in the Sangre de Cristo mountains, the Kiwanis meadow in the Sandias mountains, the South Baldy Trail meadows in the Magdalena mountains, the Hospital Flat meadow in the Pinalaño mountains, and the Barfoot Park meadows in the Chiricahua mountains (Table 1). Sampling occurred in high elevation meadow ecosystems with annual average temperatures ranging from approximately 42-76°F in June, 47-80°F in July, and 39-72°F in August. Sampling

in the meadows followed the southwestern monsoons to ensure maximum floral bloom during sampling periods (Crimmins et al. 2011).

Table 1. Meadow Descriptions. The GPS coordinates, altitude, and collection dates are listed for each meadow. The attitudes at each meadow were relatively similar to keep the sites as consistent as possible. The dates to visit each meadow were selected to optimize the blooming at each site. Collectors moved from North to South to follow the peak precipitation of the Southwest United States monsoon season.

Meadow	GPS	Altitude (m)	Dates Visited
Johnson's Mesa	36.8486, -104.2147	2083	July 10-13
Jack's Creek	35.8416, -105.6555	2727	July 16-19
Kiwanis	35.2034, -106.4430	2854	July 23-26
South Baldy Trail	33.991475, 107.183675	3115	Aug 4-7
Hospital Flat	32.668392, 109.877642	2756	Aug 10-15
Rustler Park	31.9056, 109.2798	2599	Aug 16-21

Pollinator sampling

Sampling was conducted during July and August 2012 to follow the North American monsoons as they traveled south. At each study location, a team of two spent five days in each meadow from approximately 9:00 am until 2:00 pm. Weather conditions were standardized. Pollinator species were collected in proportion to their abundances for one sampling hour each day. Each specimen was killed in a cyanide “kill jar” and kept separate from other specimens to prevent cross-contamination of pollen samples. Each kill jar was then cleaned of residual pollen before its reuse to prevent unnatural spreading of pollen between specimens. Once removed from the kill jar, each specimen was pinned and labeled with its collection site, date, and unique specimen number. The team collected over 6000 specimens, which were stored in wooden boxes with ample space between them to avoid pollen cross-contamination. The plant species each specimen was observed visiting in the field was documented and classified as observation data. Because such lengths were taken to preserve the pollen samples on each specimen, I made the assumption that each pollinator visited all of the plants from which it contained pollen grains. This assumption allows me to use the pollen grains present on each specimen to identify both how many and what types of plants it visits.

Pollinator subsample selection

After species identification, I subsampled from the thousands of specimens to determine which would be tested for pollen identification and quantification. I randomly sampled by location within each butterfly species. I verified that each species was represented for each location in which it was found by first separating the specimens by species, then subsampling by location within each species grouping. In a power analysis pilot study I determined that three specimens from a single location provided a comprehensive representation of the pollen species collected, so I randomly sampled three specimens per meadow. The random sampling of each species at each location was achieved by compiling all of the specimen labels, sorting them by species and location, and using a random number generator to randomly select from each group. In total I sampled 75 specimens from a total of 272 specimens representing 13 butterfly species.

Pollen identification and quantification

In order to extract and identify pollen samples, I used glycerin jelly slides and a high-power compound microscope and camera. First I speared a small piece of solid, stained glycerin jelly, approximately 2 mm³ in size, with a needle. For 30 seconds, I rubbed the glycerin jelly square over the body of the specimen, making sure to avoid the wings and pollen sacs. Next I placed the jelly on a clean slide and warmed it gently over a flame until the jelly melted and placed a cover slip to seal the mixture. Examining the slide under 400x magnification, I compared the pollen sample I collected to reference pollen samples collected from known plants in each meadow. For each specimen slide, I recorded both the number of different pollen species present, as well as which plant species were visited. The plant species data allowed me to determine the pollination syndrome.

Table 2. Lepidoptera species abundance and distribution. From each site in which a species was found, I randomly sampled up to three specimens collected in that meadow to test for pollen. The species with the most specimens tested for pollen was *Colias eurytheme*, as it was found in all five meadows. Conversely, *Vanessa atlanta* was only found in one meadow and consequently had the fewest specimen tested. *Vanessa cardui* was the most abundant with a total of 97 specimens collected.

	MM	SC	JC	CH	PL
<i>Colias eurytheme</i>	3	11	27	5	36
<i>Euptoierta claudia</i>	1		1	3	8
<i>Eurema nicippe</i>			2		2
<i>Limnetis breolowii</i>				6	
<i>Papilio polyxenes</i>				1	4
<i>Phyciodes tharos</i>				1	4
<i>Poanes zabulon sp. taxiles</i>		1			
<i>Polygonia gracilis</i>	8	1		2	
<i>Pontia protodice</i>			9	1	
<i>Speyeria atlantis</i>		2	1		34
<i>Vanessa atlanta</i>				1	
<i>Vanessa cardui</i>	16		3	11	62
<i>Vanessa carye sp. annabella</i>	1		1	1	2

Network structure

I analyzed pollinator-plant interactions using both the observation data and the pollen data. First I looked at the observation data collected in the field that details the plant species each specimen was collected from. That data was then compared to the pollen network data, which incorporates the pollinator-plant interactions revealed in the pollen analysis. For each network, I calculated the modularity, which measures the extent to which species have more links within a module than they would have by random chance (Bosch et al. 2009).

Specialization

To investigate the difference between the diversity of interaction partners represented by the pollen and observation data, I conducted a paired t-test. Additionally, I looked at specialization within each species using the d' statistic (d') for degree of specialization (Blüthgen et al. 2006). The degree of specialization of animals measures the degree of interaction specialization at the species level and is derived from Shannon entropy. It is often used to analyze variation within networks (Blüthgen et al. 2006). The d' statistic takes into account species abundance in its calculations, which made it ideal for my study with a wide range of pollinator richness.

RESULTS

Butterfly and pollen identification and quantification

Of the 13 Lepidoptera species (Table 2), *Colias eurytheme* was the most widespread as it was found in all five study sites. Several species, including *Vanessa atlanta*, only had one collected specimen. The study site with the greatest species richness was the Chiricahua Mountains with 10 butterfly species (Table 2). The study site with the lowest species richness was the Sangre de Cristo Mountains with five butterfly species. *Vanessa cardui* was the most abundant with a total of 97 specimens collected, and *Vanessa atlanta* was the least abundant with only one specimen collected.

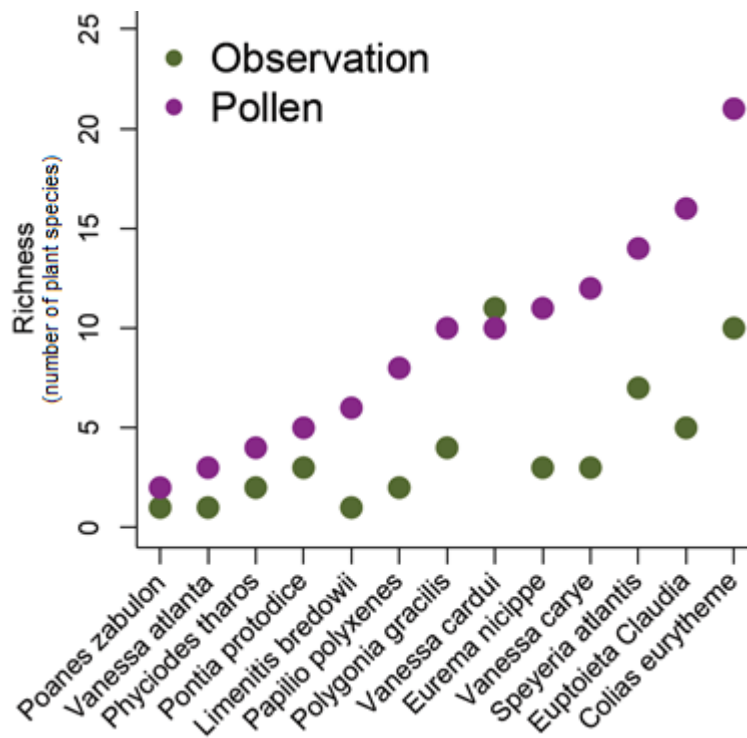


Figure 1. Plant species richness of each butterfly species. Richness is a measurement of the number of individual plant species visited by each butterfly species. With the exception of *Vanessa cardui*, the pollen data had higher species richness than the observation data across all species. Despite having fewer specimens, the pollen data was still able to connect each species with more plants.

My pollen identification yielded 38 of the 89 plant species present in the five meadows compared to the 19 plants the observation data detected. With the exception of *Vanessa cardui*, the pollen data found higher plant species richness than the observation data across all butterfly species (Figure 1). Despite having fewer specimens to analyze, the pollen data still yielded more comprehensive interaction networks.

The median plant species richness was significantly higher in the pollen data than the observation data (Figure 2). The paired t-test that compared the plant richness between the two data sets confirmed the difference in pollinator-plant connections ($t = -7.0421$, $P < 0.001$).

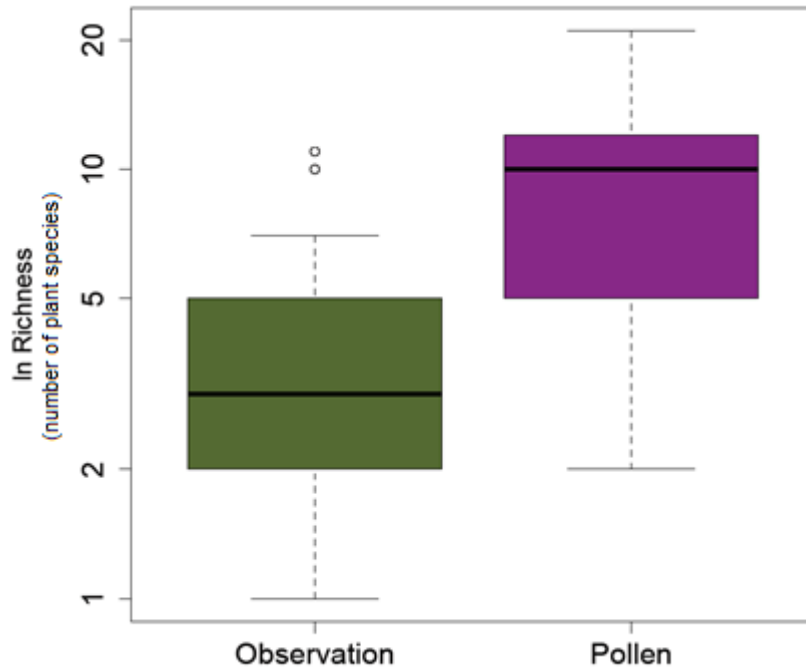


Figure 2. Plant species richness compared between assessment methods. Richness is a measurement of the number of individual plant species visited by each butterfly species. The pollen data revealed significantly more pollinator-plant interactions than the observation data ($P < 0.001$). Boxplots represent medians (black horizontal line) first and third quartiles (box perimeter) and extrema (whiskers).

Network structure

The observation data yielded 18 plant species, while the pollen data found that the 13 pollinator species visited 38 plants (Figure 3). Repeatedly comparing the networks to null models, I found a modularity score of 0.121 ($P = 0.001$). Most of the links that were discovered through visual surveys in the observation data were also revealed through the pollen analysis.

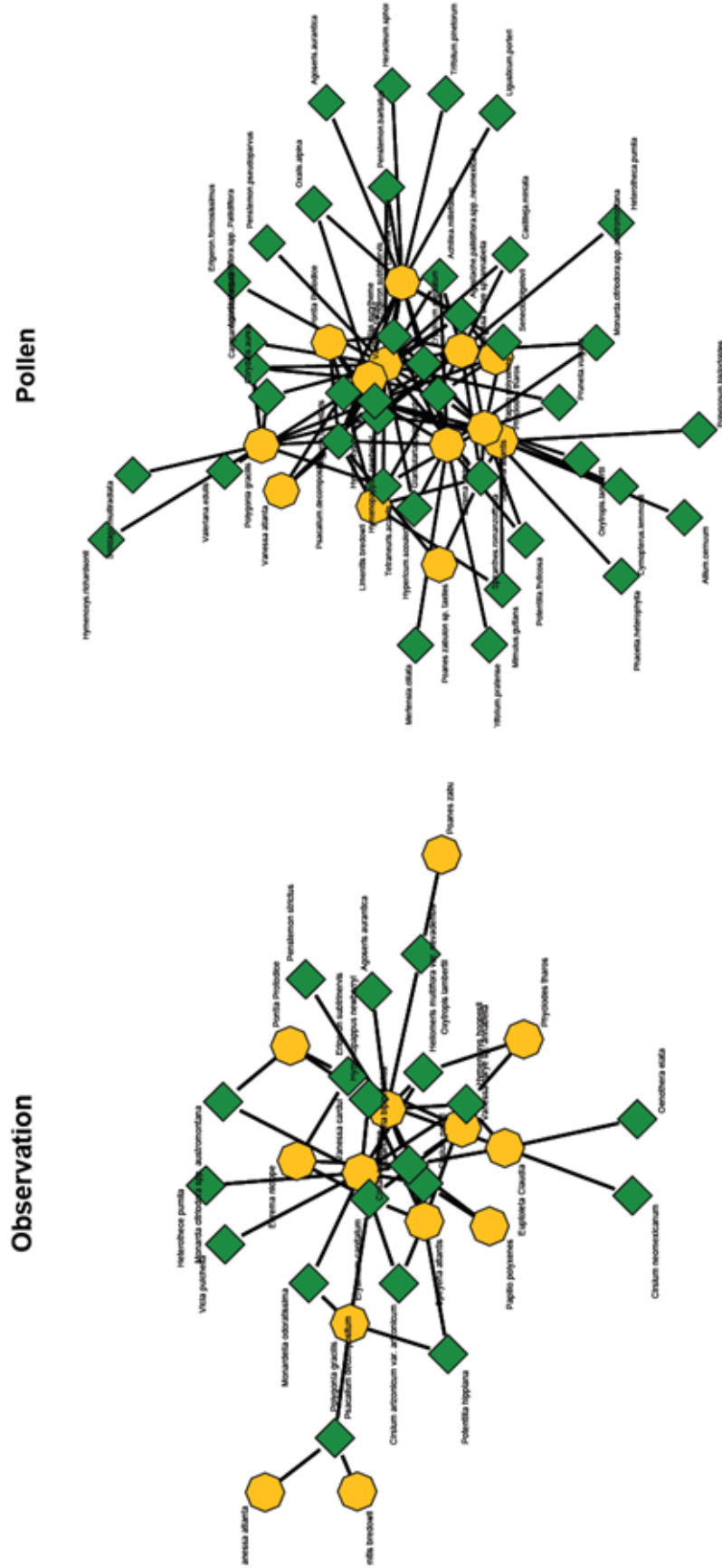


Figure 3. Network figures. Representations of the pollination networks using both the observation and pollen data. Green squares represent plant species and yellow dots represent butterfly species. The pollen data had higher interaction diversity than the observation data. The pollen data detected more pollinator-plant interactions than the observation data, and consequently has a much more complex module.



Figure 4. Interaction matrix. The modules are broken up by spaces between the plants and pollinators. Darkness of each square indicates the strength of the interaction based on its shade, with darker squares representing stronger interactions. This figure is plotting the pollinator-plant modules using only the observation data. Of all of the plant species, the butterflies showed a strong preference for *Glandularia bipinnatifida*. There were many plant species detected with the pollen data that do not appear on this figure. This representation of the observation data reveals how specialized butterflies appear without the pollen data. In comparison to the other pollinators in the collection, butterflies appear relatively specialized. Further studies could investigate the power of pollen analysis with these other more generalized pollinator species.

Further investigation into the entire collection’s modularity found the strength of interactions between specific pollinators and the 98 plants in the study sites (Figure 4). For butterflies, the strongest interaction was with *Glandularia bipinnatifida*. This interaction matrix was composed entirely of observation data interactions.

Specialization

For 8 of the 13 butterfly species, the pollen data was more generalized than the observation data (Figure 5). The abundance of each butterfly species was taken into account when calculating the d-statistic. The largest gap between observation and pollen data d- statistic values was for *Vanessa cardui*, which was also the only species with higher plant species richness in the observation data than in the pollen data.

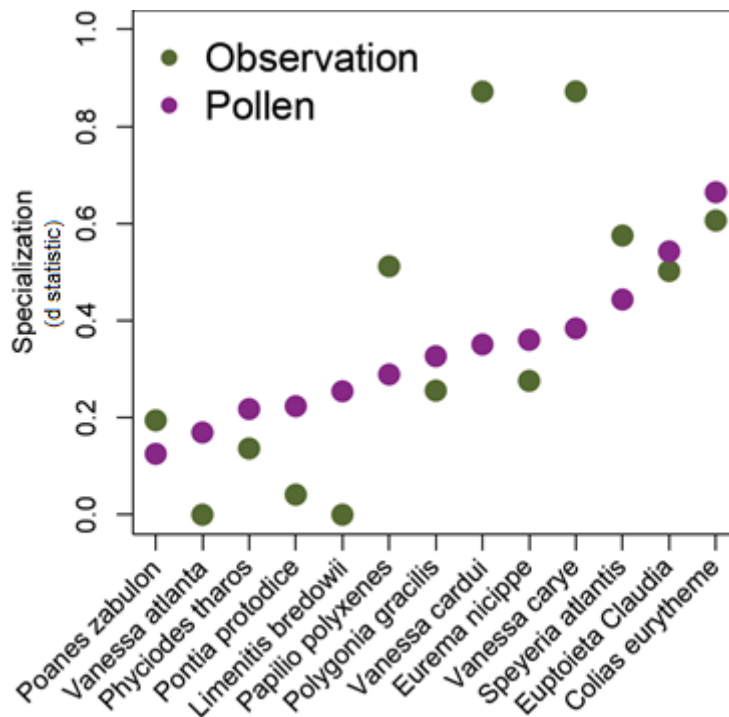


Figure 5. Specialization. Comparison of d-statistic of specialization between observation and pollen data. The d-statistic is measured on a scale from 0-1, with 0 being more specialized and 1 being more generalized. For 8 of the 13 butterfly species, the pollen data appeared more generalized than the observation data.

The median d-statistic for pollen was slightly higher than the observation data median (Figure 6). The range of the observation values was significantly greater than the pollen data values. This is most likely attributable to the wide range in number of specimens per species within the observation collection. Regardless of how many specimens were in the collection, I only sampled a maximum of 15 specimens per species. This difference in sample size may account for the different spreads.

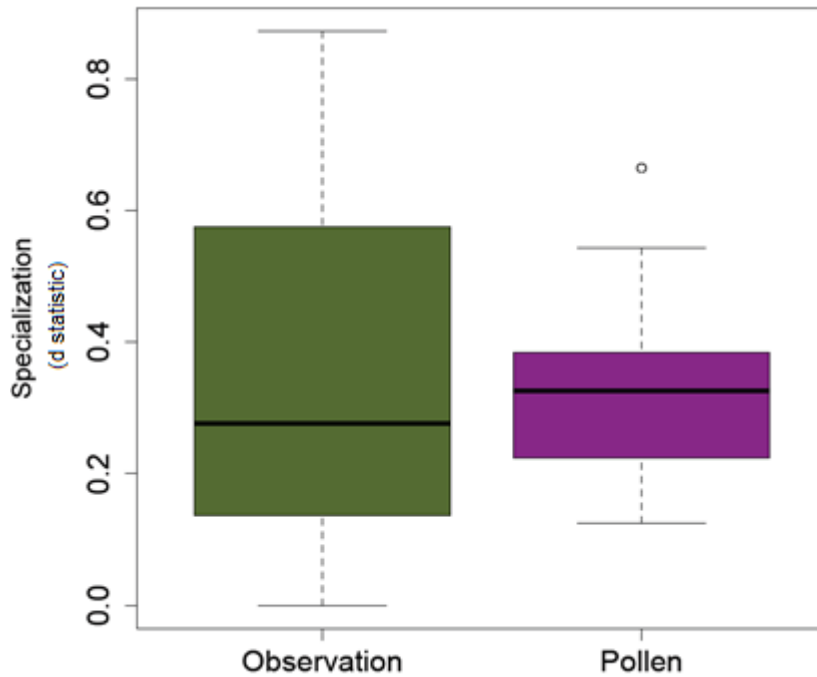


Figure 6. Butterfly specialization boxplots. Comparison of d-statistic of specialization between observation and pollen data. Boxplot interpretation is as for Figure 2. The d-statistic is measured on a scale from 0-1, with 0 being more specialized and 1 being more generalized. The pollen data indicated that butterflies were more generalized than perceived by the observation data.

DISCUSSION

Recently, scientists have been gravitating toward the theory that generalization in pollinators is more dominant than specialization. However, many observation-based studies find conflicting results. I investigated whether the incorporation of pollen analysis on the pollinators would improve our understanding of pollinator-plant interactions in order to obtain a more complete understanding of the network structure. Incorporating pollen analysis into a pollination

network study increased the number of pollinator-plant interactions observed. The pollen data allowed for the detection of low frequency interactions that would otherwise require extended observation periods in the field. There was significantly increased richness within the pollen data in comparison to the observation data despite the fact that the observation data had substantially more specimens.

Of the 89 plant species present at the five study sites, 38 were identified as pollen present on the butterfly specimen. Having 13 butterfly species allowed me to look at the addition of pollen data across multiple species networks; overall there was little variety in the number plant species visited by a single species between sites. For example, *Eurema nicippe* collected pollen from three plant species in the Chiricahua mountains, two species in the Sangre de Cristo mountains, and four species in the Pinaleño mountains. Conversely, there was considerable variety in the number of plant species visited among different butterfly species (Figure 1). While all 13 butterfly species were connected through pollen analysis to at least one plant species, some individual specimens were found to have no pollen present at all. This is a relatively common limitation of pollen analysis (Kanstrup & Olesen 2000, Forup et al. 2008), in which no pollen grains are collected off a specimen's body.

Because some butterfly species were more abundant than others, I was also able to compare the power of pollen data on rare and common butterfly species networks. Rare pollinators are often falsely categorized as specialists (Waser et al. 1996, Bosch et al. 2009), and my examination of the pollen data supported that belief of false categorization. The plant species richness of all rare pollinators increased with the addition of pollen data. Due to the large specimen collection I was sampling from, I only analyzed pollen from three specimens per species per location. Some species, such as *Vanessa atlanta* and *Poanes zabulon sp. taxiles*, only had one specimen present in the collection. This limitation may have caused an underestimation of the number of pollinator-plant interactions actually present for those species. However, these individuals highlighted the benefits of pollen analysis over observation data, because they otherwise would have only been recorded visiting one plant. Instead, my pollen analysis found that the *Poanes zabulon sp. taxiles* and *Vanessa atlanta* specimens contained two and three pollen species respectively. Even on an individual specimen level, the pollen analysis was able to provide more information on visitation behaviors than observation alone.

Modularity

The pollen data was expected to increase the plant-butterfly network links; butterflies can carry pollen loads for long periods and over long distances (Courtney et al. 1982), and consequently the pollen data was expected to provide more information on pollinator-plant interactions than would visitation data. The modularity within the pollen data was significant ($M = 0.121$), indicating a strong interlinking of butterflies and plant species within the system (Olesen et al. 2007, Bosch et al. 2009). In general, modularity and module size tend to increase with increased plant species richness (Olesen et al. 2007). Overall, the pollen data produced greater interaction diversity than the observation data (Figure 3).

Specialization

Of the 13 butterfly species, eight appeared generalized with the inclusion of pollen analysis (Figure 5). This coincides with the increasingly popular view that generalists, rather than specialists, dominate within pollination networks (Waser et al. 1996, Bosch et al. 2009, Ollerton et al. 2009). Because the majority of pollinator species were revealed to be more generalized once their pollen was examined, it is reasonable to believe that visual surveys may mislead researchers into believing pollinators tend to be specialized. However, there were five butterfly species that appeared more specialized in the pollen data than when using the observation data. The most significant difference between the pollen and observation data was for *Vanessa cardui*, one of the most abundant butterfly species. The observation data included visual surveys of 97 different specimens, while I analyzed the pollen of 14 specimens. Overall, however, the addition of pollen data provided insight on additional pollinator-plant links, which allowed for a more accurate understanding of the butterflies' generalized visitation behavior.

In general, the pollen data was more generalized than the observation data (Figure 6), suggesting that visual surveys may not provide the most accurate representation of network structure. By better understanding the number of pollinator-plant interactions through pollen analysis, we can get a better sense of the actual level of specialization found in nature.

Limitations and future directions

While my study was conclusive, additional studies involving different pollinators and ecosystems could provide further insight into the power of pollen analysis. Differences may arise due to different pollinators' characteristics. Butterflies are able to carry pollen loads for extended periods of time and across long distances (Courtney et al. 1982). However, other pollinators may have a different natural ability to maintain a collection of pollen on their bodies for shorter lengths of time, and consequently pollen data may be more or less beneficial to their network studies. Pollen analysis also does not take into account visitation frequency, which could be an important factor to consider when studying pollination networks (Bosch et al. 2009). Additionally, the meadow sites at which these butterflies were collected were all of the same ecosystem type. Therefore it would be inaccurate to assume these results would hold true in another location or with another pollinator without additional research.

There are opportunities for additional inquiries into the importance of incorporating pollen data into network analyses. Conducting a similar study with different pollinators would provide a better scope of the importance of pollen data. Additionally, looking at butterflies and other pollinators across various ecosystems is another area in need of exploration. For example, butterfly diversity varies geographically (Devries 2001); therefore looking at pollination networks closer to the equator where butterfly diversity is greatest might yield significantly different results. With additional data, the importance of including pollen analysis while studying pollinator networks can be fully established.

Conclusions

The incorporation of pollen data enhanced the connectivity of pollinator-plant networks, and as a result should be taken into consideration when conducting network studies. Although observation data provides an adequate representation of pollinator-plant interactions, a more complete network structure can be constructed with pollen data. Any study of pollinator biodiversity requires the understanding of its network structure and its responses to disturbances. Examining the modularity of the system could provide insight into how an ecosystem may react to a disturbance. Key species within the module would have a greater impact on the network, and

consequently should become high conservation priorities. Conversely, less integral pieces of the network would have smaller impacts if they were to go extinct (Olesen et al. 2009).

I found that the number of pollinator-plant connections within a pollination network increased significantly after the examination of pollen data. Butterfly species that appeared highly specialized in the observation data became more generalized after analyzing their pollen. The strong t-test significance strongly implies that pollen analysis can provide better insight into pollinator-plant interactions than can observational data alone. As a result, I determined that pollen analysis is a beneficial addition to visual surveys for pollination network analysis.

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REFERENCES

- Blüthgen, N., F. Menzel, and N. Blüthgen. 2006. Measuring specialization in species interaction networks. *BMC ecology* 6:9.
- Boggs, C. L., and C. L. Ross. 1993. The Effect of Adult Food Limitation on Life History Traits in *Speyeria Mormonia* (Lepidoptera: Nymphalidae). *Ecology* 74:433–441.
- Bosch, J., A. M. Martín González, A. Rodrigo, and D. Navarro. 2009. Plant-pollinator networks: adding the pollinator's perspective. *Ecology Letters* 12:409–419.
- Courtney, S. P., C. J. Hill, and A. Westerman. 1982. Pollen Carried for Long Periods by Butterflies. *Oikos* 38:260–263.
- Crimmins, T. M., M. A. Crimmins, and C. D. Bertelsen. 2011. Onset of summer flowering in a “Sky Island” is driven by monsoon moisture. *New Phytologist* 191:468–479.
- DeVries, P. J. 2001. Butterflies. Pages 559–573 in Editor-in-Chief: Simon A. Levin, editor. *Encyclopedia of Biodiversity*. . Elsevier, New York.
- Forup, M. L., K. S. E. Henson, P. G. Craze, and J. Memmott. 2008. The restoration of ecological interactions: plant–pollinator networks on ancient and restored heathlands. *Journal of Applied Ecology* 45:742–752.
- Inouye, D. W. 2007. Pollinators, Role of. Pages 1–9 in S. A. Levin, editor. *Encyclopedia of Biodiversity*. . Elsevier, New York.
- Johnson, S. D., and K. E. Steiner. 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution* 15:140–143.
- Ollerton, J., R. Alarcón, N. M. Waser, M. V. Price, S. Watts, L. Cranmer, A. Hingston, C. I. Peter, and J. Rotenberry. 2009. A global test of the pollination syndrome hypothesis. *Annals of Botany* 103:1471–1480.
- Pacini, E. 2008. Pollination. Pages 2857–2861 in S. E. Jorgensen and B. Fath, editors. *Encyclopedia of Ecology*. . Academic Press, Oxford.
- Vázquez, D. P., and M. A. Aizen. 2004. Asymmetric specialization: a pervasive feature of plant–pollinator interactions. *Ecology* 85:1251–1257.
- Waser, N. M., L. Chittka, M. V. Price, N. M. Williams, and J. Ollerton. 1996. Generalization in Pollination Systems, and Why it Matters. *Ecology* 77:1043–1060.
- Waser, N. M., and J. Ollerton. 2006. *Plant-Pollinator Interactions: From Specialization to Generalization*. . University of Chicago Press.