

Flying by night: Comparing crepuscular pollinator networks across two sites in Western North America

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Abstract:

Recently, declines among both honeybees and native pollinators have raised concerns about the impacts of habitat loss and climate change on pollinators; likewise, studies have revealed that non-honeybee pollinators, including various Lepidoptera, are more important than previously thought for both agriculture and the maintenance of biodiversity within native ecosystems. Such research generally focuses on diurnal pollinator networks, while crepuscular pollinator networks remain largely understudied. However, what is known about crepuscular pollinator networks is that moths account for many of the plant-pollinator interactions and thus occupy a crucial place in these networks.

There is still a deficit of information on crepuscular pollinator networks -- many of the existing studies are focused on a specific moth-plant interaction, or are geographically restricted to specific sites. This study aimed to contribute to the growing body of literature documenting crepuscular pollinator networks. Over three weeks in summer 2020 (July-August), moths were collected and sampled for pollen at sites in California and Colorado. Plant-pollinator networks were then constructed for the two sites, and compared for various network indices. The study revealed that overall these moths do not appear to be efficient or large-scale pollinators for these networks, but that they are generalists which may be visiting a wide range of flower species over larger ranges. Additionally, significant differences in the structure of these two networks suggest that crepuscular plant-pollinator networks may vary widely between different locations.

Introduction:

Pollinators play an important part in many ecosystems. Plant-pollinator interactions are known to facilitate crop set and maintain biodiversity within native ecosystems; over 40 important food crops are more productive when wild pollinators are present (Garibaldi *et al*, 2013) , and up to 85% of known angiosperms are dependent on animal pollination for reproduction (Ollerton, 2017). Recent declines in pollinator populations have led to concerns about the continued preservation of this important ecosystem service, and raised questions about the impacts of urbanization (Baldock *et al* 2015; Geslin *et al* 2013; Theodorou *et al*, 2017), land use management (Merkx *et al* 2015; Rickets *et al* 2008), and climate change on pollinator networks, as is attested by an increasing number of studies devoted to documenting pollinator declines and elucidating the causes behind them (Garibaldi *et al*, 2013; Ollerton, 2017).

But a survey of the literature reveals that many of these studies may be missing an important part of the picture. Much more is understood about the roles and population changes of the European honeybee than any other pollinator, due to its role in human agriculture (Ollerton, 2017). When studies do examine wild and native pollinators, there is once again a taxonomic bias -- wild bees are more likely to

be researched than pollinators like Lepidoptera, wasps, and hoverflies (Ollerton, 2017). However, it is clear that crepuscular pollinators, and particularly Lepidoptera, are important. Studies on cloudberry and lowland blueberry fruit set have shown that nocturnal pollinators contribute more to fruit set than previously assumed (Cutler *et al.*, 2012). Research on specific moth-flower interactions, such as specialized orchids, suggests that moths can also be critical for preserving rare flowering species which may be entirely dependent on one species of moth for propagation (Pemberton, 2010). They can also be generalists that visit a wide variety of blooms in complex networks, as was shown in a study which found that the pollination of less-common flora is subsidized by hawkmoths' reliance on agave inflorescences (Alarcon, Dawidowitz, and Bronstein, 2008).

From a conservation standpoint, it is just as valuable to understand how moths fit into plant-pollinator networks. Studies on crepuscular pollinator networks as a whole, though uncommon, have illuminated some aspects of the central role of moths as pollinators after dark (Hahn & Brühl, 2016). Moths account for the majority of nocturnal pollination recorded, though different studies report different percentages of moths carrying pollen in a given network (from 3% up to 75%) and a wide range of pollen load, in terms of grain count (Banza, Belo, & Evans 2015, Devoto *et al.* 2011, Hahn & Brühl, 2016). In some cases these differences have been recorded within studies conducted over several seasons; in other cases, the studies in question were conducted in different locations (Hahn & Brühl, 2016). Either way, even with the gradually accumulating number of studies devoted specifically to crepuscular and nocturnal plant-pollinator networks, there remains much to learn.

This study aimed to address this research gap by surveying crepuscular pollinator networks in two locations -- Gunnison County, CO and Santa Clara County, CA. At field sites in both locations, moths were collected and sampled for pollen grains to construct plant-pollinator networks and record moth diversity. The two locations differed significantly in habitat composition. The Santa Clara County, CA study site is near sea level and is close to a large urban center; the Gunnison County, CO study site is above 9,000 feet and is close to large wilderness areas. It was hypothesized that the composition of plant-pollinator networks and the diversity of moths present at these studies would be significantly different,

due to differences in locality. The secondary question this study hoped to answer was how specialized moths in both study sites are to local flora. Studies have shown that many butterfly and some moths species have co-evolved with flowers that they rely on; among other factors, this is evident in matching proboscis lengths and flower corollas (Johnson, 2010). The benefit to the pollinator is in decreased energy expenditure while foraging; flowers are thought to benefit when individual flowers have longer-than-average corolla tubes, increasing the probability that pollen is deposited on moths. Over the course of the study, moth proboscis length was measured to determine whether mean proboscis length differs between species and sites. Differences in mean proboscis length could indicate that the diets of sampled species may be specialized towards corresponding flowers.

Methods:

Fuchsin jelly and slide preparation:

The fuchsin jelly was prepared using 175 mL distilled water, 300 mL glycerin, 50g gelatin, and crystals of basic fuchsin (Beattie, 1971). (The amount of glycerin required to set the jelly was higher than that recommended by Beattie.) The gelatin was added to the distilled water in a beaker and heated until the gelatin dissolved; then the glycerin was added, along with small amounts of fuchsin crystals, and the resulting solution filtered through cheesecloth. The fuchsin jelly was then refrigerated or cooled until set, as needed. When set, the fuchsin jelly was cut into 0.5x0.5mm cubes to be used for swabbing.

Once the cube had been used to collect pollen (from flowers or moths), the cube was placed on a glass slide, melted over a candle flame, then covered with a glass cover slip and sealed around the edges with clear nail varnish.

Constructing pollen reference library:

At all field sites, flower samples were collected from any blooming, white flowers within a 75m radius of the site; the plants were photographed for later identification, and flowers collected in plastic bags. Pollen

samples were gathered from the flowers by rubbing the anthers onto preprepared fuchsin jelly, then treating the jelly as described above.

Moth sampling:

In Gunnison County, Colorado, sampling occurred in two locations -- at a house in Washington Gulch, and at a cabin at Rocky Mountain Biological Laboratory. In Santa Clara County, California, sampling occurred in two locations at Foothills Park -- next to Boronda Lake, and next to the Nature Interpretive Center. CA sites were sampled five times each over the course of 3 weeks; the Washington Gulch, CO site was sampled four times, and the RMBL, CO site was sampled once. Sampling occurred between 9:00pm and 11:15-30pm at CA sites, and between 9:00pm and 12:00am at CO sites. At CA sampling locations, a white sheet was strung up with a blacklight shining on it in order to attract moths. At CO sampling locations, the moths were collected from the surfaces of lit windows. When a moth landed, it was captured and cooled on ice until it could be handled. (For ease of identification, and because they are less likely to be pollinators, microlepidoptera were not sampled.) After at least 15 minutes on ice, the moth was swabbed for pollen on the thorax, head, proboscis, and maxillary palps. Pollen samples were preserved using fuchsin jelly, as described above. Proboscis length was measured using electronic calipers and recorded, as was the sex of the moth and the amount of wing wear (as a proxy for age). The moth was photographed to be later identified using iNaturalist and other resources available on the Internet. If the moth was a species that had not yet been collected, it was kept and pinned as a voucher specimen. If one or two individuals of the species had already been captured for pinning, the moth was marked with a silver dot on the underside of the thorax and released. Voucher specimens from CO sites were sent to CA to be pinned; slides from CO were also sent to CA for processing.

Pollen grains were counted and identified using the pollen reference library. A USB microscope (magnification x400 - x1000) was used for counts, and for obtaining photographs of pollen to compare against the pollen reference library. When samples were unable to be identified, the pollen was compared to outside pollen reference libraries. Additionally, when fungal spores were seen on slides, the presence of

fungal spores was noted and fungal spores were photographed to be later identified by an outside resource.

Data analysis:

For proboscis length, an ANOVA test was used in R to compare mean proboscis length across species, and a t-test to compare mean proboscis length between California and Colorado. For calculating species richness, the “betadivers” function in the R package “vegan” was used; the “specaccum” function was used to calculate species accumulation curves. The R package “bipartite” was used for construction of pollinator networks at both sites; the “null.t.test” function, with $N = 100$ iterations, was used in order to determine the significance of network indices for networks at each site. Moth species were included as nodes when at least one individual of the species was found to be carrying at least one pollen grain; moths and pollen grains that have not been identified were included in the network construction. For comparing binomial data (moths carrying pollen and moths carrying spores), a Chi-squared test was performed.

Results

Pollinator specialization

Proboscis length was measured for all collected species CA and CO, when possible. The proboscis was not measured for species on which it could not be located -- *Malacosoma incurvum* and species 8 in CO, and *Ostrinia penitalis*, *Nadata gibbosa*, species 114, and species 22 in CA. The proboscis length was also not measured on some moths that were released before measurement -- individuals 20 (*Drasteria edwardsii*), 60 (*Drepanulatrix bifilata*), 80, 109, and 118 (*Drepanulatrix bifilata*). The means for proboscis length in all species in both California and Colorado were calculated and compared using an ANOVA test; the difference in mean proboscis length was significant ($F_{49, 52} = 15.9$, $p < .001$) (Fig. 1). Mean proboscis length for all individual moths, regardless of abundance, was then compared between California and Colorado using a t-test. Mean proboscis length in California was 4.7 mm, and in Colorado, 6.3 mm; a t-test showed that the difference in mean proboscis length was significant ($p < 0.001$, $df 101$) (Fig. 2). There was no significant difference in means for the two sites in California (results not shown).

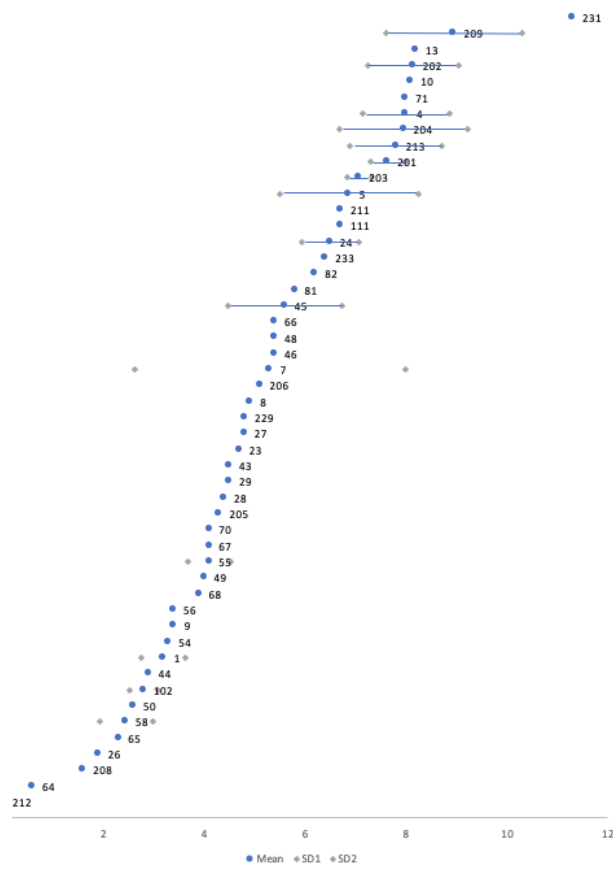


Figure 1: Mean and standard deviation for proboscis length in mm, by species. Species numbers “200” and up are from Colorado; all others are from California. Points without standard deviations were species for which only one individual had been sampled. Differences between species were significant ($p < .001$, $df 101$) with an ANOVA test.

Network metrics

A total of 48 different moth species was sampled at CA; a total of 15 different species was sampled from Washington Gulch, CO (RMBL sample data forthcoming). Given that moths were sampled over 10 nights in CA, and only four nights in CO, beta-diversity was used to normalize species diversity by sampling intensity; however, even when normalized against sampling intensity, moth diversity in CA

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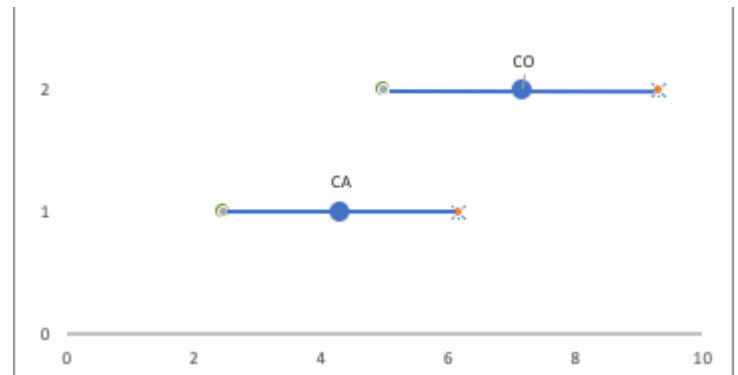


Figure 2: Mean and standard deviation for proboscis length in mm, by site. Mean proboscis length for moths in CA was 4.7 mm; mean proboscis length for moths in CO was 6.3 mm. Difference between means is significant ($p < 0.001$, $df 101$).

appeared higher than in CO -- 0.85 compared to 0.65. Moth species were undersampled at both sites; for a better estimate of moth diversity, more sampling dates would be needed (Fig. 3).

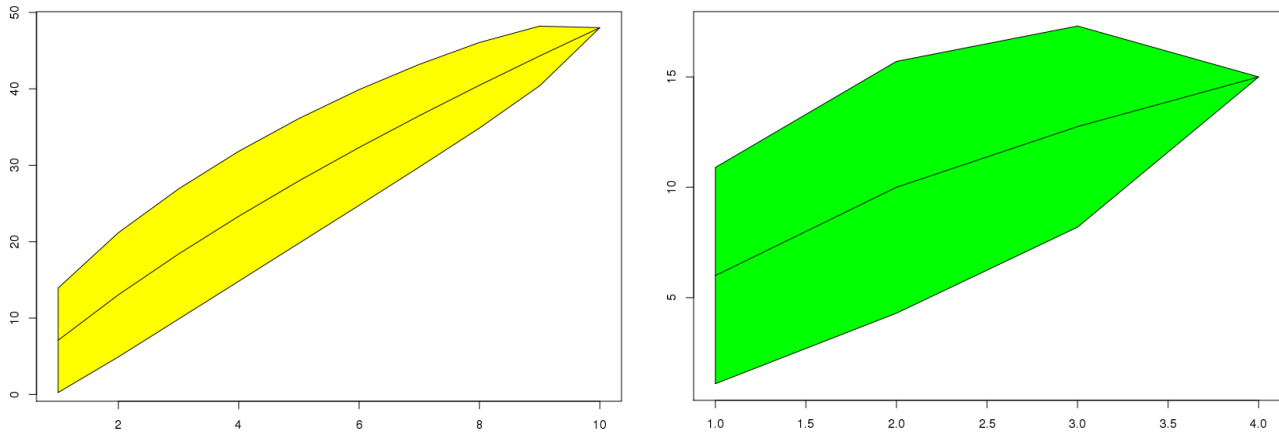


Fig. 3: Species accumulation curves for CA (left) and CO (right). X-axis represents nights of sampling; Y-axis represents species count, and yellow colors represent the confidence interval for number of species sampled. Neither curve reaches its asymptote.

Due to timing constraints, only 45 of the 118 swabbed moths from California were analyzed for pollen counts. The moths arbitrarily selected for this analysis were individuals 60 through 118; 45 of these moths were used for all analyses related to pollen type and pollen carry counts. The percentage of moths carrying pollen and not carrying pollen was compared using a Chi-square test for both of the sites. The proportion of moths carrying pollen in Colorado was significantly higher than in California ($\chi^2 = 7$, $df = 1$, $p = 0.009$) (Table 1). Even moths that were carrying pollen tended to carry small amounts -- in California, the mean pollen load for individuals carrying pollen was 2.17 with a mode of 1, while the mean pollen load for individuals in Colorado was 3.5 with a mode of 1. Both of these means are still well below 5 pollen grains, the threshold for biological significance suggested by Devoto et al (2011). In California, 4.4% of all moths scanned for pollen were found to be carrying 5 or more pollen grains; in Colorado, 9.8% of moths were found to be carrying 5 or more pollen grains -- a higher number, but not statistically so (Table 1). Additionally, many moths were observed to be carrying fungal spores, mostly in the order Pleosporales (known plant pathogens); fungal spore load was not quantified, due to time constraints and difficulty obtaining accurate counts of spores.

Network metric	Foothills Park, CA	Washington Gulch, CO
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Proportion of moths carrying pollen	17/45* (37.8%)	28/41* (68.3%)
Proportion of moths carrying > 5 pollen grains	2/45 (4.4%)	4/41 (9.8%)
Number of species carrying pollen grains	14	9
Number of species (plants)	9	16
Connectance	0.14**	0.29**
Linkage	0.78**	1.68**
Nestedness	8.40**	47.82**
Specialization	0.77**	0.48**
Niche overlap (pollinators)	0.124**	0.25**
Robustness (pollinators)	0.621**	0.68**

Table 1: A comparison of some pollinator network metrics. * indicates $p < 0.01$ significance between sites; ** indicates $p < 0.01$ significance between the network metric and null models based on the network for a given site.

While the number of moth species collected at CA was higher than at CO, the number of different plants that moths interacted with was higher at CO than at CA. Connectance ($0.14 < 0.29$), linkage ($0.78 < 1.68$), nestedness ($8.40 < 47.82$), niche overlap ($0.124 < 0.5$), and robustness ($0.621 < 0.68$) were all higher for the CO network than the CA network, though network robustness was relatively similar; the CA network, however, had a higher specialization ($0.77 > 0.48$) (Table 1, Figure 4). Connectance is a measure of the percent of existing links compared to all possible links; linkage is a measure of the mean number of links between organisms; nestedness is a measure of the degree to which species with many interactions interact with species that have few interactions; niche overlap is a measure of similarity in interaction pattern between species of the same trophic level; robustness is a measure of network resistance to the

removal of species; and specialization is a measure of the degree to which species are consistent in interaction choices, rather than opportunistic (Dormann, 2020).

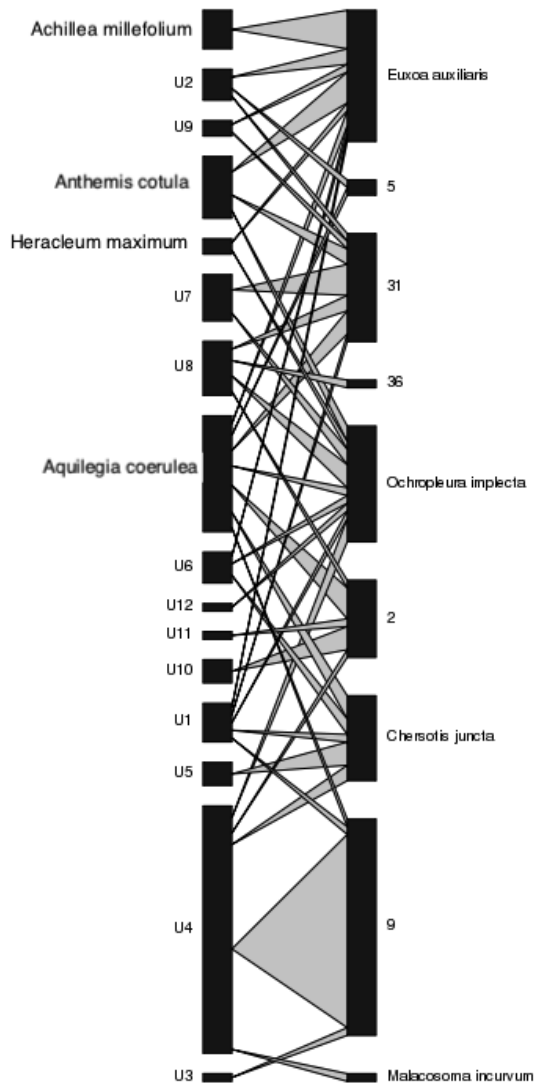
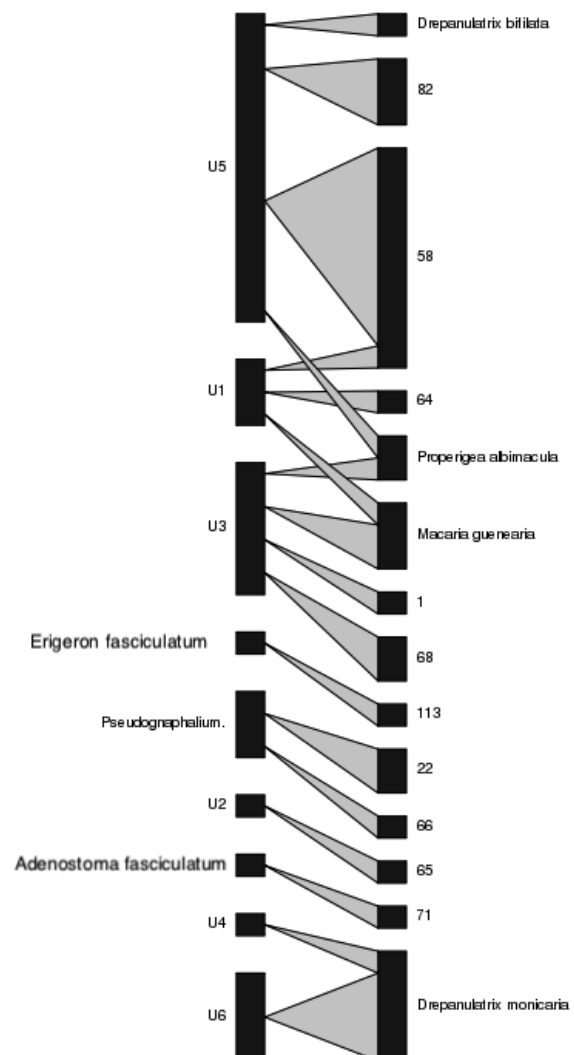


Fig.

4: Pollinator networks in CO (left) and CA (right) field sites. Black rectangles represent species (plant species on the left, moths on the right), while gray bars represent interactions (pollen grains from the plant were found on the moth.) The width of the bars corresponds to the proportion of pollen grains of a given species found on the moth. Latin names were provided where possible; numbers represent species that have not yet been identified, for both plants and moths.



Discussion

The preliminary results of this study suggest that there is diet specialization occurring among moths in CA and CO -- there were significant differences in mean proboscis length when all species of collected moths were compared, and mean proboscis length was on average smaller in moths collected at California compared to moths in Colorado. This may partly reflect differences in moth diversity; moth species richness was much higher in California than in Colorado, even when differences in sampling intensity were accounted for, and many of the non-Noctuid species in California had smaller proboscides than the Noctuidae in Colorado. It is possible that the California study site had a higher diversity due to elevational differences; the California site is near sea level, while the Colorado site is above 9,000 feet. The greater richness of trees, and therefore the greater richness of food sources for moth larvae, at this elevation in California may lead to the observed difference in moth diversity.

Our findings that moths play a relatively small role from the perspective of pollen load correspond to other studies with similar methods (Clinebell *et al* 2004, Devoto *et al.* 2011, LeCroye *et al* 2013). 4.4% of moths in California carried a significant pollen load, and 9.8% of moths in Colorado. So few moths were found carrying a biologically significant load, with only six individuals across the whole study carrying more than 5 pollen grains, that it was necessary to include individuals carrying non-significant loads in order to construct networks. The proportions of moths carrying a biologically significant pollen load in CA and CO still fall within the range found by other studies, which could be anywhere from 3% in a Scottish boreal forest in 2017 to 76% in southwestern Portugal (Banza, Belo, & Evans 2015, Devoto *et al.* 2011). On the other hand, there was a high diversity of moth-plant interactions, especially in the Colorado network, where more plant species were recorded in the network than moth species and connectance was relatively high. Likewise, in both networks, many types of pollen grains could not be identified, and therefore were not from white-blooming within 75 meters of the study sites. This suggests, as some other studies have (LeCroy 2013), that the pollination services of moths may have more of an impact through long-distance dispersal of pollen, rather than quantities of pollen transferred.

While this study was not focused on relationships between fungal spores and moths, the high prevalence of fungal spores -- particularly the spores of fungi such as that are known to be plant pathogens -- on most of the sampled moths suggest that there are interactions happening between them. Without identification of the spores found on these moths, it is difficult to say what these results might indicate. Some studies have shown that moths may disperse plant pathogens; it has been shown that in greenhouses, moths can transfer spores from infected to healthy sorghum (Prom *et al* 2003), although the presence of moths with fungal conidia is not necessarily correlated to the prevalence of fungus in the habitat (Butler *et al* 2001). It is possible that moths are dispersing these fungal spores in CA and CO, but it is unclear what impact this may be having on local levels of plant infection; more intensive research would need to be done in order to draw any conclusions.

The networks in Colorado and California both had distinct network indices. Connectance, linkage, nestedness, robustness, and niche overlap were all higher in Colorado, where fewer moth species were pollinating more varieties of flowers. Moths in California were more specialized. This may be explained in part by the greater presence of Noctuidae in Colorado samples, which may all be visiting similar flower varieties compared to the more specialized and varied moths in California. While it is difficult to draw any conclusions from such a small study, the lower robustness of the California network may suggest that from a conservation standpoint, it is more critical to maintain habitats with a wide variety of wildflowers that will support its greater moth diversity than in Colorado. What these network indices do indicate is that findings for one crepuscular pollinator network are not necessarily applicable to other crepuscular networks. In order to obtain a functional understanding of the traits of a crepuscular pollinator network in a given ecosystem, such studies are critical.

This study was under many constraints which could be biasing results. The time period of sampling was limited to three weeks over one summer; previous studies conducted over multiple years have shown that there can be great temporal variability in the prevalence of moth species and their roles as pollinators within a network (Devoto *et al* 2011). The method and timing of collection may also have impacted results -- not all moth species fly to light; light traps may attract moths before they have visited

flowers and gathered pollen loads; and moth collection never occurred after 12:00am at either site, where other studies have found that some Noctuidae flight peaks after midnight (Ollerton 2017, Clinebell 2004). Species diversity was certainly undercounted for California, where several moths were seen but not captured. Finally, the moths were swabbed on the head, maxillary palps, and thorax, while the abdomen and legs were not being swabbed; because the same piece of gel was used for swabbing all body parts, no distinction could be made between pollen load on different body parts in later analysis. A different study which compared pollen load on the head/mouthparts and the rest of the body (thorax, abdomen, legs, and wings) found that pollen load on the body was generally higher (LeCroy 2013). Were this study to be repeated earlier in the season in California, when more flowers are blooming, or at a different time, or with more thorough swabbing of moths for pollen, or with sampling at flowers in addition to light traps, it is possible that the results would change.

Previous crepuscular plant-pollinator network studies have been conducted at other sites in the USA and beyond, and previous studies have been conducted on the diversity and prevalence of moths in the CA Bay Area and Gunnison Basin is already known. However, there have not as of yet been studies conducted specifically with the goal of determining the links and network characteristics of moths and flowers at either site. Though limited in scope, this study provides a starting point for a better understanding of crepuscular pollinator networks at these two locations, and provides a groundwork for asking further questions about how the species interactions may change over time during a season, or how urbanisation and land use may be affecting moth diversity, or the impacts climate change may be having on crepuscular pollinator networks.

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