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The structure and robustness of nocturnal Lepidopteran pollen-transfer networks in a Biodiversity Hotspot

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Abstract. 1. The role of nocturnal moths within plant-pollinator networks is poorly understood but could be important in the context of declining biodiversity and the ecosystem services they provide.

2. For the first time, this study examined the role of moths as pollen vectors in the Mediterranean Biodiversity Hotspot. Light traps were used to sample moths in SW Portugal in 2010. The pollen on moth head parts was collected, identified, and counted to construct a nocturnal pollen-transfer and flower-visitor network.

3. A total of 257 moths belonging to 95 species were captured in 11 trapping sessions in 2010; 196 moths (76%) carried pollen and the total number of pollen grains counted and identified was 9064.

4. The pollen-transfer network exhibited a high degree of selectivity (H2′) but low robustness when the most-to-least connected plants were made extinct in the network. The flower-visitor network (based on the incidences of interactions by individual moths), however, exhibited high linkage density and was generally more robust to simulated plant or moth extinction.

5. Including nocturnal moths in plant–pollinator networks will provide a better understanding of their robustness to species extinctions due to environmental change as well as the impacts on ecosystem structure and functioning. Nocturnal pollen–transfer networks could be developed for identifying key species for targeted conservation.

Key words. Ecological network analysis, food-webs, interactions, Mediterranean region, moths, plant–pollinator networks, pollen transport.

Introduction

Ecological networks describe the interactions between species, the underlying structure of communities and the function and stability of ecosystems (Montoya *et al.*, 2006). In recent years, a burgeoning interest in insect–flower interactions has provided valuable insights into the structuring of mutualistic networks (Memmott, 1999; Bascompte *et al.*, 2003; Fontaine *et al.*, 2006; Memmott *et al.*, 2007), their robustness to species extinction (Memmott *et al.*, 2004; Kaiser-Bunbury *et al.*, 2010; Pocock *et al.*, 2012) as well as the impacts of environmental change (Fortuna & Bascompte, 2006; Lopezaraiza-Mikel *et al.*, 2007; Memmott *et al.*, 2010; Evans *et al.*, 2013). With pollinating insects experiencing significant declines in many parts of the world (Biesmeijer *et al.*, 2006; An &

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Chen, 2011; Meeus *et al*., 2011), there is a pressing need to better understand the impacts on insect–flower interactions (Morandin & Winston, 2006; Klein *et al*., 2007) and the consequences for ecosystem functioning and services.

Globally, fruit, vegetable, or seed production from 87 of the leading global food crops is dependent upon animal pollination (Klein *et al*., 2007). The importance of Lepidoptera as pollinators within natural, unmanaged ecosystems are well documented as is their co-evolutionary relationships with plants (Proctor *et al*., 1966; Kevan & Baker, 1983; Nilsson, 1998; Fenster *et al*., 2004; Schiestl & Schlueter, 2009), but little is known about their importance for pollinating crops (Macgregor *et al*., 2015). Moreover, whilst the drivers adversely affecting butterfly declines and extinctions are understood (Thomas *et al*., 2004), we know relatively little about the factors affecting moth populations (Fox *et al*., 2014), which is important given that nocturnal moths represent around 95% of all Lepidoptera species (Winfree *et al*., 2011).

To date, nearly all studies of plant–insect pollinator interactions have focused on diurnal pollinators (Memmott, 1999; Dicks *et al*., 2002; Bascompte *et al*., 2003; Memmott *et al*., 2004; Bascompte & Jordano, 2007; Olesen *et al*., 2007, 2008; Bosch *et al*., 2009; Ings *et al*., 2009) but a recent study suggests that overlooked nocturnal pollinators might also be important for ecosystem functioning (Devoto *et al*., 2011). The role of moths in communities is linked not only to plant reproduction (as mutualists) but also as plant herbivores (as antagonists, particularly during larval stages) as well as their trophic importance for organisms higher in the food web (Macgregor *et al*., 2015). Ultimately, understanding the role of moths in communities is essential for the conservation of both moths and the organisms that depend on them, either for pollination or as a food source, especially in biodiversity rich parts of the world where many elaborate mutualisms have co-evolved (Bawa, 1990).

The Mediterranean Basin is an important 'Biodiversity Hotspot', with over 22 500 endemic vascular plant species (of which approximately 52% are endemic); more than four times the number found in all the rest of Europe (Blondel & Aronson, 1999; Medail & Quezel, 1999). Despite its importance for biodiversity, we know relatively little about nocturnal plant–pollinator interactions in this region or the importance of moths as ecosystem service providers. Moreover, as one of Europe's major holiday destinations, populations of threatened species are likely to become increasingly fragmented as a result of resort and infrastructure development, although detailed studies are lacking.

In this study, we examine for the first time the importance of moths as pollinators in SW Portugal, part of the Mediterranean Biodiversity Hotspot. Our objectives are threefold: (i) to determine the proportion of nocturnal moth species caught that carry pollen and are therefore likely involved in pollination processes; (ii) to identify the main species of moths and plants involved in these processes, and (iii) to construct and analyse the structure and 'robustness' (a measure of the tolerance of the network to species extinctions, Dunne *et al*., 2002; Memmott *et al*., 2004) of a quantitative nocturnal moth-pollen-transfer network. We compare our results to other pollen-transfer networks and discuss the implications for the conservation of species interactions, ecological processes and ecosystem services.

Materials and methods

Field site

The study was carried out in an abandoned meadow (approximately 360 m^2) in the Western Algarve as part of the Ria de Alvor Natura 2000 Site (37°07′–37°09′N and 08°35′–08°38′W). The Alvor estuary is an important area of wetlands, dunes and farmland protected from the sea by two sand spits, which shape the beaches of Alvor and Meia Praia. The estuary is at the confluence of three tributary streams, forming a lagoon system around two peninsulas – Quinta da Rocha and Abicada. It is the most important wetland area in the Western Algarve in terms of size and conservation status and the third most important in the Algarve (Jorge & Kaye, 2001).

Vegetation sampling

We characterised the plants that were in flower that were likely nectar sources for moths within the study area in 2010. Vegetation was sampled in 20 quadrats, systematically set in a 4 × 5 line grid arrangement separated by 15 m. We used the Braun-Blanquet method for vegetation sampling but only recorded plants that were in flower (as a potential source of nectar for feeding moths). We recorded plant species and abundance, vegetation cover (%), and height (cm). The site was sampled on 19th and 24th March, 6th and 21st April, 5th and 18th May, and 3rd June 2010 to correspond with moth sampling sessions (see below). We constructed a pollen reference collection from the flowering plants at the study site. During each vegetation survey, a sample of a new flowering plant was collected, placed into a separate plastic bag, identified and labelled for pollen collection. Pollen was extracted from the flower and fixed on to a microscope slide using fuchsin jelly. The slides were kept for later observation to compare and identify the pollen transported by moths (see below). Nomenclature followed Flora Europaea (Tutin *et al*., 1964–1980). Where necessary, the nomenclature of the species was updated according to Flora Iberica (Castroviejo *et al*., 1986–2009).

Vegetation sampling sessions coincided with moth sampling sessions to adequately describe all flowering plants within the study area that the moths could potentially interact with (see Supporting Information for the list of all plant species recorded). During the study, it became necessary to collect additional pollen samples (in a

non-systematic manner) from other plant species found in the wider locality of the study site (i.e. within a 500 m radius) to identify the diversity of pollen carried by moths (see below). Where necessary, pollen identification was facilitated by the use of a pollen collection from Évora University and specialised literature (Smith, 1984; Abreu & Moreno, 1998; Boi & Llorens, 2007). In most cases, identification of pollen was to the level of species, with the exception of *Urtica* sp., *Pinus* sp., *Cupressus* sp., *Acacia* sp., *Plantago* sp., *Prunus* sp. to genus level and Poaceae to the family level.

Moth sampling

The dates of the moth sampling sessions were chosen specifically having taken into account the local weather forecast and the brightness of the moon to trap as many moths as possible. Rainy and cold nights and/or a bright moon are associated with fewer numbers of moths trapped in this locality (P. Banza, pers. obs.). Moth trapping sessions were conducted on 19th March, 7th, 21st, 29th April, 7th, 11th, 18th, 26th May, and the 3rd, 10th, 24th June in 2010.

We used a standard, portable 6W UV-light (Philips TL 6W/05, Philips, Poland) heath trap (placed on top of a white sheet) to attract and capture moths. The trap was set on the ground in the centre of the field at sunset (20:00) and collected the next day at sunrise (07:00). Captured moths were placed into individual tubes and frozen prior to processing in the laboratory, following Devoto *et al.* (2011). In the laboratory, moths were identified using a reference collection from 'A Rocha Portugal' and appropriate guides (Waring *et al.*, 2003; Manley, 2008). They were then swabbed for pollen using a circle of fuchsin jelly to determine which plant species had been visited and to construct pollen transport networks (Forup & Memmott, 2005; Forup *et al.*, 2008; Bosch *et al.*, 2009). The area of the head between the base of the antennae, the labium and the eyes was swabbed, as this is the area of the body most likely to touch plant reproductive structures while feeding. Whenever possible, the proboscis was uncoiled and swabbed as well. The fuchsin jelly was melted onto a microscope slide and kept for later pollen identification and counting. To avoid fungal contamination a fine layer of colourless nail varnish was used to seal the slide content.

Constructing the nocturnal plant-pollinator networks

Information on the quantity and identity of the pollen carried by each moth species from the study site was pooled to build a quantitative pollen-transfer web representative of the habitat. Although our assumption is that moths are transferring pollen between plants, we acknowledge that this is not necessarily the case for all species as demonstrated by studies on diurnal flower-visitor networks (Popic *et al.*, 2013). Interactions between plants and moths were only included in the analysis when at least five pollen grains from the same plant species were counted in the pollen load of a single moth. This was taken as evidence that the moth actually visited that particular plant species and reduced the potentially biasing effect of pollen contamination, which may have occurred: (i) due to heterospecific pollen transfer by visitors between co-flowering plant taxa, (ii) in the light trap, and/or (iii) subsequent handling of the moths (Devoto *et al.*, 2011). Pollen identification from wind-pollinated species (e.g. *Pinus* spp., *Olea europea* L., *Cupressus* spp., *Eucalyptus globulus* Labill., *Acacia* spp., *Casuarina* sp.) was not considered for the network analysis because the pollen grains from those plants carried by the moths do not represent a pollen transfer – they are anemophilous and not entomophilous. When an individual slide contained more than 100 pollen grains ($n = 2$), a subsample was taken and the total number of grains was estimated. The pollen grains from undetermined plant taxa ($n = 5$) were not considered for the network analysis.

For a better understanding of community interactions we constructed both a pollen-transfer network and a flower-visitor network. The first network includes the total number of pollen grains carried by each moth species, the proportion of pollen of each plant taxa carried by each moth species and the total number of pollen grains of each plant taxa from all individuals captured (Devoto *et al.*, 2011). The second includes the total number of moths of each species that interacted with a plant using the same pollen threshold (i.e. five or more grains) for an interaction to have occurred (note: a flower-visitor network based on presence/absence of pollen and not using the threshold is in Figure S1 in the Supporting Information). In effect, it is an animal-focused way of constructing a plant-pollinator network based on the incidence of pollen occurring on moths, rather than the traditional approach considering the number of insects visiting plants. We assessed the sampling completeness of moths, pollen/plants and species-interactions by plotting species accumulation curves and the Chao 2 estimator of asymptotic species richness (that uses a non-parametric method to estimate species richness based on the concept that rare species carry the most information about the missing ones) following Chacoff *et al.* (2012).

We examined the structure and complexity of the networks using commonly used metrics calculated by the function 'network level' from the package 'bipartite' in R 3.0.2 (Dormann *et al.*, 2009). We determined moth species and plant taxa richness, links per species (sum of links divided by number of species), linkage density (marginal totals-weighted diversity of interactions per species), selectivity of the whole network [an index of 'complementarity specialisation', that is, the selectivity in the use of resources measured as H2′; Bluthgen *et al.* (2006)], interaction evenness [based on Shannon diversity of interactions; Bersier *et al.* (2002)], and weighted nestedness [NODF; Almeida-Neto *et al.* (2008); Almeida-Neto and

Ulrich (2011)]. We also examined the robustness of the networks to simulated species extinctions using the functions 'second.extinct', 'robustness' and a modified version of 'slope.bipartite' from package 'bipartite'. Although sampling biases are likely to affect network-level analyses such as this, our aim was to provide the first measure of the 'fragility' of the networks for the region. First, we simulated the sequential loss of moths carrying pollen and then recorded the proportion of plants still remaining, calculating robustness as the area under the curve (Burgos *et al*., 2007). If $R \rightarrow 1$, this is consistent with a very robust system in which, for instance, most of the plant species survive even if a large fraction of the animal species go extinct. Conversely, if $R \rightarrow 0$, this is consistent with a fragile system in which, for instance, even if a very small fraction of the animal species are eliminated, most of the plants lose all their interactions and go extinct. Our assumption in the models is that plants will become extinct once they have lost all of their moth pollinators, although we acknowledge that this ignores the full range of potential interactions including diurnal pollinators. The order of extinction for the higher trophic level was based on (i) pollen abundance (largest-to-smallest) and (ii) the most-to-least connected animals. The latter is the most extreme case, where the most generalist species go extinct first (see Memmott *et al*., 2004). Similarly, we examined the robustness of the network to plant extinction. Second, we simulated the sequential loss of animals and plants from the flower-visitor network (based on incidences of interactions, rather than pollen loads) as above. This was to compare the robustness of the two networks based on total pollen loads versus incidences of interactions by individual moths.

Results

Plants

During the sampling period, 50 plant taxa were identified within the study site. An additional 13 plant taxa were recorded within a 500-metre circle from the field site. The most abundant species were *Pallenis spinosa* (L.) Cass., *Daucus carota* L., *Scorpiurus muricatus* L., *Euphorbia* spp. (mainly *E. exigua* L. and *E. helioscopia* L.) and *Sherardia arvensis* L. The plant species flowering during the entire period of field work were *Centaurea pullata* L., *Stachys arvensis* L. and *Euphorbia exigua* L. The species present for a short period of time were *Bellardia trixago* L., *Melilotus indicus* (L.) All., *Leontodon taraxacoides* (Vill.) Mérat, *Ornithogalum narbonense* L., *Trifolium* spp. and *Linum tenue* (Desf.).

Moths

Overall, a total of 257 moths from 95 different species were captured in 11 trap-nights during the sampling period. The total number of pollen grains counted and identified was 9064 from 196 individual moths (i.e. 76% of individuals caught). The average pollen load per individual moth was 46.3 grains based on the total number caught. From those, 97 moths carried a significant amount of pollen (i.e. five or more pollen grains of at least one plant taxon – 38% of the total) which was used to construct the networks (see below). Using this threshold, 6177 pollen grains carried by 97 individual moths were included in the network analysis, with an average pollen load of 63.7 grains per moth. Of these, 58 species carried five or more pollen grains from 27 plant taxa (not including wind-pollinated plant species). It was not possible to identify three micro-moth species carrying pollen and they were excluded from the network analysis. For some of the moths identification was possible only at the genus level (i.e. *Cnephasia* sp., *Agdistis* sp.). We found a statistically significant correlation between the number of individuals caught and the number of plant taxa in the pollen load ($r_s = 0.634$, $P < 0.001$).

Pollen transfer and nocturnal flower-visitor networks

The analysis of sampling completeness suggested that we were successful in sampling approximately 68% of plant/pollen species, 42% of moth species and 11% of the interactions. The pollen-transfer network consisted of 27 plant and 58 moth species (Table 1) but was dominated by pollen from the two plant species *Anagallis arvensis* L. and *Cynoglossum creticum* Mill. (Fig. 1a, species C and J respectively) and was mainly carried by *Ephestia parasitella* (Staudinger 1859) (Fig. 1b, species 30) and *Eudonia lineola* (Curtis 1827) (40). Six moth species, *Eudonia lineola* (species 40), *Tyta luctuosa* (Denis & Schiffermüller 1755) (species 80) *Cleonymia baetica* (Rambur, 1837) (11), *Athetis hospes* (Freyer, 1831) (70), *Pterolonche lutescentella* (Chrétien, 1922) (71), *Scopula marginepunctata* (Goeze, 1781) (74) carried pollen from over eight different plant species. The network had 1.64 links per species, a linkage density of 2.382 and an interaction evenness of 0.29 (Table 1). The network tended towards complete selectivity ($H2' = 0.79$) with a weighted NODF of 15.97 (Table 1). Sequentially deleting the most to least connected species in

Table 1. Descriptors of network structure and complexity of the nocturnal moth species-interaction networks, SW Portugal.

	Pollen-transfer network	Flower-visitor network
No. of moth species	58	58
No. of plant taxa	27	27
Links per species	1.64	1.64
Linkage density	2.38	8.07
Interaction evenness	0.29	0.65
Weighted NODF	15.97	21.93
$H2'$	0.79	0.15

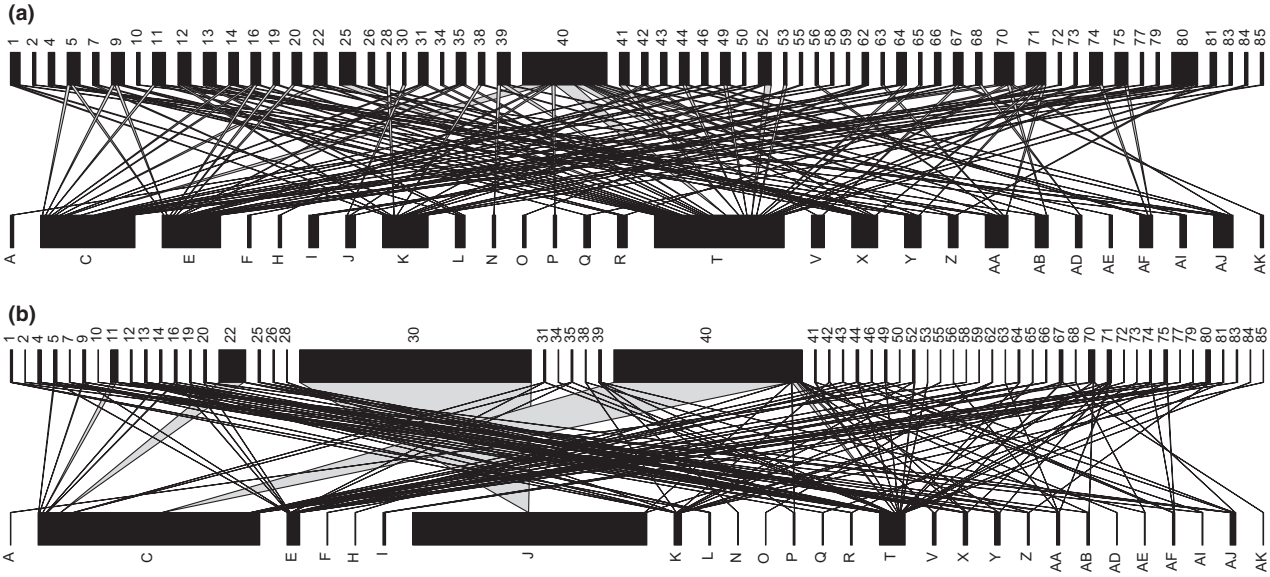

Fig. 1. Moth pollen-transfer networks from SW Portugal using (a) data based on the number of moths carrying pollen from different plants and (b) data based the total number of pollen grains transfered. Each black rectangle represents the proportional abundance of moths and pollen/plants and each grey triangle represents the frequency of interaction between the moths and pollen/plants. See Supporting Information for the complete list of all flowering plants and moths.

the pollen-transfer network resulted in robustness values of 0.54 for moths (Fig. 2a) and 0.32 for plants (Fig. 2c) respectively. Sequentially deleting the least to most abundant species resulted in robustness values of 0.82 for moths (Fig. 2b) and 0.86 for plants (Fig. 2d).

The flower visitation network had a higher linkage density of 8.07 and interaction evenness of 0.65 (Table 1). The network had low selectivity ($H2' = 0.15$) with a weighted NODF of 21.93 (Table 1). Sequentially deleting the most to least connected species in the flower visitor network resulted in robustness values of 0.55 for moths (Fig. 2e) and 0.32 for plants (Fig. 2g) respectively. Sequentially deleting the least to most abundant species resulted in robustness values of 0.87 for moths (Fig. 2f) and 0.89 for plants (Fig. 2h).

Discussion

Our study revealed that a significant proportion (76%) of the nocturnal moths we caught carried pollen. This is a considerably higher proportion than the 8% of individuals recorded carrying pollen in Scottish pine forests, the only other nocturnal moth pollen-transfer network study we are aware of (Devoto *et al*., 2011). Although caution is needed when distinguishing between flower 'visitors' and 'pollinators' (King *et al*., 2013), our results suggest that moths may be important, but overlooked, pollinators in the Mediterranean region, warranting further research. The pollen-transfer network metrics reflect the dominance of a small group of species. Here, the network showed high selectivity and was dominated by two moth species, *Eudonia lineola* and *Ephestia parasitella*, which carried most of the pollen load. Approximately one-third of the species caught carried pollen from five or more plant species. Despite potential sampling biases (see below), we found a significant correlation between the number of specimens of each species caught in the field and the number of plant taxa found in the pollen loads, suggesting that generalist pollinators are likely to be the most abundant species in this region. From an ecological network perspective, this result supports the assumption that species abundance and ecological generalisation (measured as the number of different interaction partners) are highly correlated (see Vazquez *et al*., 2007), but is contrary to the relationship observed by Devoto *et al*. (2011), perhaps due to the fact that a much smaller proportion of moths trapped in northern Scotland carried pollen. Using the incidences of pollen found on moths to construct flower-visitor networks for nocturnal moths is a novel method for understanding species interactions that might otherwise be difficult to observe in the field. Our moth flower-visitor network was highly robust to simulated species loss as moths were generally linked to a high number of flowering-plants.

Limitations

The main limitation of this study is that it was conducted at a single site, although this is not unusual in network ecology (Memmott, 1999; Lopezaraiza-Mikel *et al*., 2007; Pocock *et al*., 2012; Evans *et al*., 2013). Furthermore, our sampling completeness was low. At our study

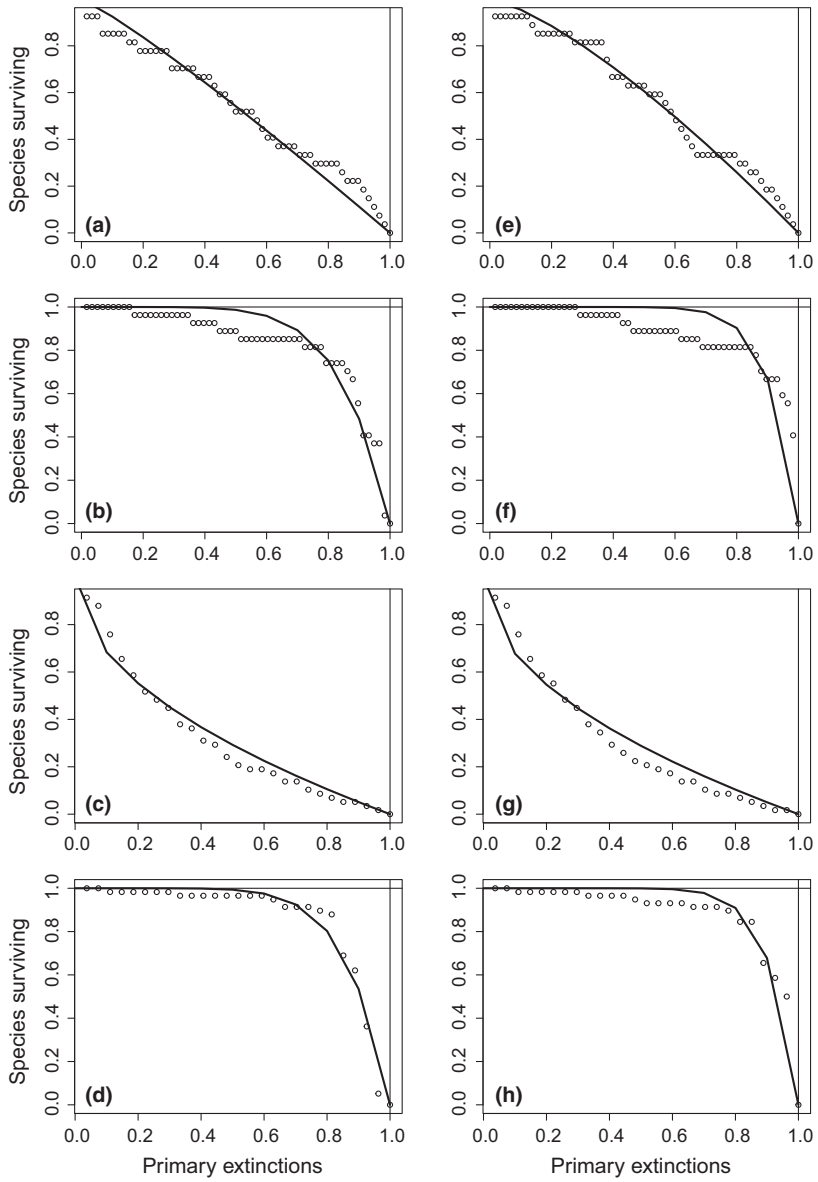

Fig. 2. The robustness of the pollen-transfer networks to simulated species extinction using (a–d) quantitative data that include moths carrying five or more pollen grains and (e–f) qualitative data based on the incidence of moths carrying pollen. Using pollen-transfer data, the figure shows the sequential loss of: (a) the most-least connected pollen; (b) the least-most abundant pollen; (c) the most-least connected moths; (d) the least-most abundant moths. Using flower-visitor data, the figure also shows the sequential loss of: (e) the most-least connected plants; (f) the least-most abundant plants; (g) the most-least connected moths; (h) the least-most abundant moths.

site, in the majority of cases only one individual of each moth species was caught, making it difficult to draw any conclusions about their relative importance as pollen vectors and thus the network metrics need to be treated with caution. We believe the results of this study will open new areas of research examining the importance of moths as pollinators within the Mediterranean biodiversity hotspot. Temporally and spatially replicated nocturnal networks will be necessary to determine the generality of our results, as in diurnal networks (Olesen *et al*., 2008).

Another limitation of this study is the bias inherent in the use of light traps. Light traps are widely used to attract nocturnal moths, because they result in large numbers of specimens being caught with a minimum of effort (Beck & Linsenmair, 2006). Light traps, however, produce a biased sample of the community because they measure activity rather than abundance, and because attraction to light varies among species (Macgregor *et al*., 2015). Running transects or using timed observations, as carried out in diurnal flower-visitor studies, is potentially one way of

overcoming this problem (Birkinshaw & Thomas, 1999), although more research is necessary.

Moth pollen-transport and flower-visitor networks

Mutualistic networks share some common properties such as the presence of many specialists but few generalists (Waser *et al*., 1996; Jordano *et al*., 2003; Devoto *et al*., 2011) and often a nested pattern of interactions (Bascompte *et al*., 2003). Our pollen-transfer network contained considerably more plant and moth species than the Devoto *et al*. (2011) study in Scotland, and showed higher selectivity ($H2' = 0.79$, cf. 0.38), although there was variation between years. However, when we compare these results with the flower-visitor network properties, it appears that the latter does not show high selectivity in the use of resources measured ($H2' = 0.12$). Instead, the network is characterised by higher linkage density. We suggest that creating a visitation network using pollen data is an additional method for understanding species-interactions in pollination ecology (even if it does not tell us whether or not an insect is actually pollinating a plant).

The conservation of species-interaction and ecological processes

Ecological networks have the potential to quantify the effects of human activities on a wide range of complex ecological interactions (Memmott *et al*., 2007; Tylianakis *et al*., 2008). Recent work has shown that plant-pollinator network structure can be altered by global environmental change drivers, and that these alterations may have important ecosystem-level consequences (Tylianakis *et al*., 2010). Our results suggest that there might be more ecological redundancy in plant-pollinator networks than previously thought if nocturnal moths do indeed provide an important functional role as pollinators, which has implications for more complete network analyses (such as robustness) and the conservation of species-interactions. Studies showing effects of anthropogenic changes on the structure of interaction networks (Lopezaraiza-Mikel *et al*., 2007; Memmott *et al*., 2007), even when species richness is unaffected (Tylianakis *et al*., 2007) lend support to previous calls for the conservation of network structure (McCann, 2007; Tylianakis *et al*., 2010). In Portugal, there is very little information regarding population trends of moths because, as a whole, this group of insects has been insufficiently studied to provide even basic presence/abundance and distribution data. Moreover, very few studies have examined the functional importance of moths in the Mediterranean region. This is important given the increasing recognition of the ecosystem services provided by pollinating insects. Despite the limitations, we believe that the results of this study will renew an interest in the functional importance of plant–moth interactions and enable a better understanding of ecosystem functioning more generally. An ecological network approach can provide useful information not only about the distribution and abundance of moth species, but can also be used to help understand potential causes of decline and the fragility of interaction networks to species loss. Furthermore, this approach has the potential to be developed to understand and plan the restoration of plant-pollinator networks (Devoto *et al*., 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: doi: 10.1111/icad.12134:

Figure S1. A matrix of plant–moth interactions based on the presence/absence of pollen identified on moths (this data does not use the 5 + pollen threshold as used in the main text). Plant codes (A-AL) and Moth codes (1-85) can be found in Table S1. The matrix shows the nested structure of the plant–moth network, with darker shades of grey representing a greater number of observed interactions.

Table S1. The plant and moth species identified in the study site, SW Portugal. Codes refer to Fig. 1 in the main text.

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