

How biased is our perception of plant-pollinator networks? A comparison of visit- and pollen-based representations of the same networks

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Natasha de Manincor, Nina Hautekèete, Clément Mazoyer, Paul Moreau, Yves Piquot, et al.. How biased is our perception of plant-pollinator networks? A comparison of visit- and pollen-based representations of the same networks. Acta Oecologica, 2020, 105, pp.103551. 10.1016/j.actao.2020.103551. hal-02942290

HAL Id: hal-02942290 https://hal.science/hal-02942290

Submitted on 5 Nov 2020

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- How biased is our perception of plant-pollinator networks? A comparison of visit- and pollen based representations of the same networks
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22 Abstract

Most plant-pollinator networks are based on observations of contact between an insect and a flower 23 in the field. Despite significant sampling efforts, some links are easier to report, while others remain 24 unobserved. Therefore, visit-based networks represent a subsample of possible interactions in which 25 the ignored part is variable. Pollen is a natural marker of insect visits to flowers. The identification 26 27 of pollen found on insect bodies can be used as an alternative method to study plant-pollinator interactions, with a potentially lower risk of bias than the observation of visits, since it increases the 28 29 number of interactions in the network. Here we compare plant-pollinator networks constructed (i) from direct observation of pollinator visits and (ii) from identification of pollen found on the same 30 insects. We focused on three calcareous grasslands in France, with different plant and pollinator 31 species diversities. Since pollen identification always yields richer, more connected networks, we 32 focused our comparisons on sampling bias at equal network connectance. To do so, we first 33 compared network structures with an analysis of latent blocks and motifs. We then compared 34 species roles between both types of networks with an analysis of specialization and species 35 positions within motifs. Our results suggest that the sampling from observations of insect visits does 36 not lead to the construction of a network intrinsically different from the one obtained using pollen 37 found on insect bodies, at least when field sampling strives to be exhaustive. Most of the significant 38 39 differences are found at the species level, not at the network structure level, with singleton species accounting for a respectable fraction of these differences. Overall, this suggests that recording 40 41 plant-pollinator interactions from pollinator visit observation does not provide a biased picture of 42 the network structure, regardless of species richness; however, it provided less information on species roles than the pollen-based network. 43

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Keywords: motifs; mutualistic networks; pollen analysis; pollen network; species roles; visit
network.

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48 Data accessibility: The data analysed during the current study will be available in Zenodo upon
49 acceptance or at the reviewers' request.

51 **1. Introduction**

Plant-pollinator interaction networks are critical to the maintenance of ecosystems (Ashworth et al., 52 2009; Bronstein et al., 2006; Memmott, 2009; Vázquez et al., 2009). Pollinators indeed provide an 53 54 invaluable service, on which much of current agriculture depends (Deguines et al., 2014; Gallai et al., 2009; Klein et al., 2007), and they maintain genetic diversity in plant populations (Kearns et al., 55 1998). Reciprocally, wild plants provide various resources to pollinators, usually food and other 56 type of nutrients, hence maintaining pollinator populations (Bronstein et al., 2006; Kearns et al., 57 1998; Ollerton, 2017). Understanding the structure and functioning of these networks (i.e. how 58 59 species interact and how these interactions shape species abundance dynamics) and obtaining more 60 accurate information on plant-pollinator networks are among the current important goals of ecology. Thus, it is essential to manage and maintain insect pollination - which constitutes an 61 62 ecosystem service of global importance – because disruption of interactions can affect the diversity, 63 abundance and distribution of both plants and pollinators, with cascading consequences affecting the whole network (Gill et al., 2016). Most plant-pollinator networks are based on direct 64 observations of contact between an insect and a flower in the field. However, some links are 65 biologically (i.e. morphologically) or temporally (i.e. phenologically) forbidden, while other links 66 can remain unobserved (Olesen et al., 2011). Thus, such visit-based networks can only represent a 67 subsample of all possible interactions. Alternative methodologies or more intense sampling can 68 reduce the probability of missing some existing interactions. One such alternative method is the 69 70 identification of pollen found on pollinator bodies.

71 Pollen is a major attractant for many pollinators since it is an important part of their diet (Kearns and Inouye, 1993). Moreover, it can favour long-term associative learning in wild bees (Muth et al., 72 73 2016), influencing the floral choice of pollinators and their foraging strategy (Somme et al., 2015). As a result of this "visitation activity", *i.e.* when pollinators visit a flower, pollen becomes attached 74 75 to their bodies. Thus, it becomes a natural marker indicating the recent history of pollinator visits (Jones, 2012a) since a significant part of the pollen grains generally stay on the pollinator's body. 76 77 The identification of this pollen provides valuable information on the spectrum of pollen resources 78 and it is an important method to elucidate the foraging behaviour and the floral preferences of wild 79 pollinators, such as solitary and social bees (Beil et al., 2008; Carvell et al., 2006; Fisogni et al., 80 2018; Marchand et al., 2015), hoverflies (Lucas et al., 2018a; 2018b, Rader et al., 2011), butterflies 81 and other pollinators (Macgregor et al., 2019; Stewart and Dudash, 2016). Pollen is also often used 82 to assess pollinator effectiveness both at the community level (Ballantyne et al., 2015; King et al., 2013; Willmer et al., 2017) and at the individual level (Marchand et al., 2015; Tur et al., 2015). 83 84 Indeed, not all the visits recorded in the field correspond to actual pollination (King et al., 2013; Popic et al., 2013) and not all the pollinators are equally efficient. For example, not all pollen grains
transported by corbiculated-bees are available for the pollination event, since the moistening (using
nectar) may cause physiological changes in the pollen grain (Parker et al., 2015).

The identification of pollen found on insect bodies can be used as an alternative method to study 88 plant-pollinator interactions (Jones, 2012b). This methodology can provide a more extended history 89 record of plant-pollinator interactions than the observation of visits. Moreover, observing pollen 90 grains rather than visits removes some of the sampling biases associated with short sampling 91 92 periods and can provide an alternative view to the 'plant's perspective' provided by the observation of visits (Bosch et al., 2009; Gibson et al., 2011; King et al., 2013). However, few studies have 93 compared visit-based networks to pollen-based ones (Alarcón, 2010; Bosch et al., 2009; Olesen et 94 95 al., 2011; Pornon et al., 2017, 2016), mostly because the identification of pollen grains is timeconsuming and depends on the availability of experts with skills in palynology. The precision of 96 97 pollen identification depends on knowledge of the floral community in the study sites (Westrich and Schmidt, 1990), thus suggesting that the use of a complete pollen atlas of the co-flowering species 98 99 of the study site, as we used in the present study, may enhance the precision of identification. An 100 alternative method to microscopic identification that recently garnered interest is the use of DNA 101 barcoding (Bell et al., 2019, 2017; Macgregor et al., 2019; Pornon et al., 2017, 2016; Richardson et al., 2015). It is, however, a recent methodology not widely used in the study of plant-pollinator 102 networks and it can have some limits (Bell et al., 2017; Macgregor et al., 2019). 103

104 Various studies have pointed out that when pollen information is used to build networks, the 105 number of links between plant and insect species significantly increases, revealing changes in the network structure (Bosch et al., 2009; Pornon et al., 2017). However, all these studies compared 106 107 network structure using classic network metrics, such as connectance, nestedness and modularity, 108 which are strongly affected by network dimensions (i.e. the number of species and realised links among them; Rivera-Hutinel et al., 2012; Staniczenko et al., 2013). Thus, differences obtained in 109 the network structure when visit- and pollen-based networks are compared are essentially due to the 110 higher number of species and new links recorded in the latter. To our knowledge, only few studies 111 (Popic et al., 2013; Pornon et al., 2017) used a null model approach to take into account differences 112 in network size when comparing pollen- and visit-based networks. They found that network 113 114 structure does not significantly change between the two methods but did not investigate changes at the species level. 115

116 The aim of this study is to understand to what extent networks obtained using pollinator visit 117 records can introduce biases in the representation of the network when compared to those obtained

through pollen identification. For a constant sampling effort, we could expect that richer 118 communities are more likely to be undersampled than poorer communities. Then, the addition of 119 pollen information can lead to changes in the network structure and species roles (i.e. the set of 120 positions occupied by a species in the network), since apparent specialised species may be more 121 generalist than observed and thus separate groups of species may be more connected, revealing a 122 biased picture of the network. To test these hypotheses, we used simulated networks mimicking the 123 124 ones obtained through observation of insect visits but based on the pool of possible interactions 125 given by the pollen-based network. In a sense, these randomized networks can be considered as different "virtual observers" sampling from all the possible interactions detected using the pollen on 126 insect bodies, but with a sampling effort equal to that used in the field. This technique allowed us to 127 128 compare two networks of the same size and to check for congruence between networks. Armed with this methodological framework, we studied the plant-pollinator networks encountered in three 129 130 different calcareous grasslands in three different regions in France. We compared the two types of networks (visit- and pollen-based networks) using a new methodological approach combining 131 132 different analyses. First, we compared the network structure using latent block models (LBM) and motif analyses (Leger et al., 2015; Simmons et al., 2019a). Second, we compared species 133 specialisation level (Blüthgen et al., 2006) and species roles, based on the frequency of species 134 positions within motifs (Simmons et al., 2019a). 135

136 **2.** Materials and methods

137 2.1. Study sites and plant inventories

In this study we recorded interaction between wild bees and native herbaceous plant species in three 138 139 calcareous grasslands located in three different French regions (Fig. A.1): one in Hauts-de-France (Regional natural reserve Riez de Noeux les Auxi, noted R, 50°14'51.85"N 2°12'05.56"E), one in 140 Normandie (Château Gaillard – le Bois Dumont, noted CG, 49°14'7.782"N 1°24'16.445"E) and one 141 in Occitanie (Fourches, noted F, 43°56'07.00"N 3°30'46.1"E). We chose calcareous grasslands since 142 they are characterised by highly diverse plant communities with a high proportion of entomophilous 143 species (Baude et al., 2016; Butaye et al., 2005; WallisDeVries et al., 2002). The three sites are 144 145 three protected areas of 1 hectare each, which are included in the European NATURA 2000 network. We sampled wild bees and we recorded their interactions with flowering species during 146 147 one-day sessions in the month of July 2016 (see paragraph 2.3 for sampling details). Flowering plants were identified at the species level in the field and their abundances recorded. All plant 148 149 inventories were performed by the same two surveyors to avoid biases.

150 2.2. Pollen atlas

During each field session we sampled plant anthers of all species flowering within the study site. 151 We put the anthers in individual Eppendorf tubes filled with 70% ethanol to preserve them. From 152 this collection, we prepared a pollen atlas representative of the pollen diversity present in the three 153 areas. In the laboratory, we extracted and transferred the pollen released by anthers in the 154 Eppendorf tube on a microscopic slide mounted with a cube of glycerine jelly (Kaiser's Glycerol 155 Gelatine for microscopy) to maintain the natural colour of the pollen grains, and we sealed the 156 cover slip with nail varnish. For each slide we recorded the plant species and the site and date of 157 158 collection, and we photographed the pollen grain as reference.

159 2.3. Direct observations of plant-pollinator interactions in the field

For this study, we recorded plant-pollinator interactions for three days (one day per site) in the 160 month of July 2016, since it was one of the richest months in terms of plant and pollinator diversity. 161 Surveys of plant-pollinator interactions were performed under suitable weather conditions for 162 pollinators (following Westphal et al. 2008). The surveyors (from 4 to 5 at each session) walked 163 slowly and randomly within the site (following a variable transect as explained in Westphal et al. 164 165 2008) and hand-net sampled all wild bees visiting open flowers, recording the observed plantpollinator interaction. The sampling period consisted of 4 hours split into 2 hours in the morning 166 167 (about 10am-12am) and 2 hours in the afternoon (about 2pm-4pm), to cover the daily variability of pollinator foraging behaviour (Vaudo et al., 2014) and flower communities. All sampled insects 168 169 were immediately put into individual killing vials with ethyl acetate and were later prepared and pinned in the laboratory for identification at the species level by expert taxonomists. For some 170 171 individuals we recorded and attributed several interactions, since they were observed interacting 172 with more than one plant species before we were able to collect them.

173 2.4. Bee pollen load analysis

We focused on wild bees (superfamily: Apoidea, clade Anthophila) because, with their hairy bodies and their ability to quickly fly from one flower to another, they are one of the most efficient pollinator groups worldwide and many herbaceous plants and wild flowers depend on them for their reproduction (Ballantyne et al., 2017; Michener, 2000; Stavert et al., 2016). Moreover, wild bees present different specialized structures for pollen collection which allow them to transport large amount of pollen to feed their larvae (Alarcón, 2010; Michener, 2000). Pollen was collected from the bodies of female bees and prepared on two different microscope slides as follows: one slide with

the pollen passively transported on the body (scattered pollen, PS), and the other slide with pollen 181 actively collected in specialised structures (i.e. curbiculae or scopae, PC). Since we want to 182 compare the information provided by PS and PC slides, we only used female bees because males 183 lack adapted structure to carry pollen, such as corbiculae or scopae, (and hence cannot provide PC). 184 We collected PS from insect bodies using a small cube of glycerine jelly (volume 2 mm³) following 185 Kearns and Inouye (1993). PC was removed by brushing the specialised structures with a small 186 needle or a small spoon and put in an Eppendorf tube filled with 70% ethanol for conservation. 187 188 Only a fraction of PC (10 µl) was used to prepare pollen slides. We prepared a total of 782 pollen slides, considering both types of pollen and with information on sampling date, hour and site 189 (Fourches 346 slides, Chateau Gaillard 256 slides and Riez 180 slides). Pollen identification was 190 191 performed at the lowest taxonomic level (at species level in 90% of the cases) by an expert (K. Bieri at the Biologisches Institut für Pollen analyses, Kehrsatz, Switzerland) using a combination of 192 193 diagnostic keys and comparison with the pollen atlas described above. When it was not possible to discriminate between two closely related species, we aggregated them at higher categories (family, 194 195 genus or morphotype). Microscope slides were observed at 400x magnification by random transects 196 until we counted 100 pollen grains, then the rest of the slide was searched for undetected pollen types. However, for the statistical analyses we did not consider plant species for which we detected 197 \leq 5 pollen grains per bee individual, which we considered as infrequent or accidentally collected 198 (Bosch et al., 2009; Fisogni et al., 2018). 199

200 2.5. Characterisation of plant-pollinator interactions

To understand whether and how pollen added new links to the fieldwork observations, we separated recorded plant-pollinator interactions in five categories: (i) interactions observed as visits that were confirmed by both pollen types (PS and PC); (ii) interactions detected only by observing both types of pollen but not as visits (PS+PC); (iii) interactions found only with PS; (iv) interactions found only with PC; and (v) interactions only observed as visits but not confirmed by pollen.

We divided plant species in three groups: (a) plant species which were present in the study area (and included in the botanic inventory) and that were visited by pollinators; (b) plant species which were present in the study but whose interaction with pollinators was detected only by pollen analysis; (c) plant species present only in the surroundings of the study sites but not within them (and whose interaction with pollinators was detected only by pollen analysis). Plant species which were present in the study area but were never visited by pollinators and whose pollen we did not find on the insect bodies (*i.e.* plant species with no interactions) were excluded from the analysis altogether, and group (c) was not used for the purpose of comparing networks obtained from direct
observation of visits and pollen identification. Results of this classification were represented using a
heat map (function *quilt.plot* in R, see Supplementary Information Fig. A.2, A.3, A.4).

Prior to conducting the rest of the analyses, we tested whether the information provided by the two types of pollen (PS and PC) was different. To do so, we compared the two interaction-based rarefaction curves (Fig. A.5) using a Wilcoxon test. We found that there was not significant difference in the number of observed links between PS and PC, even if the percentage of unique links was higher for PS than for PC (results not shown). Thus, we decided to merge the information given by the two pollen types and we further refer to them as "pollen-based network" in the following analysis.

223 2.6. Plant-pollinator network analysis

We constructed two weighted (*i.e.* quantitative) bipartite networks including all pairs of interacting plant and insect species (i) directly observed as visits in the field ("visit-based" network) or (ii) retrieved from the pollen found on insect bodies ("pollen-based" network).Overall, we built three visit-based networks and three pollen-based networks (using the two types of pollen – PC and PS – found on insects), one for each site.

Raw networks were weighted networks accounting for the intensity of interactions between species pairs – in the case of visits, intensity equals the number of recorded visits of the focal pollinator species on the focal plant species; in the case of pollen identifications, intensity equals the number of insects of the focal pollinator species found with at least 5 pollen grains from the focal plant species. For some analyses (connectance, motifs and position analyses) we transformed weighted networks into binary ones.

For both binary "visit-based" and "pollen-based" networks, we calculated its *connectance* as the proportion of observed links divided by the number of all possible links. We also calculated the specialization index H2' of the weighted networks (Blüthgen et al., 2006), using the *H2fun* function implemented in the bipartite package (Dormann *et al.* 2009; R Core Team 2018).

To model compartmental structure within networks, we applied latent block models (LBM) to each network, visit-based, simulated or pollen-based. We used the *BM_poisson* method for Poisson probability distribution implemented in the *blockmodels* package (Leger et al., 2015) to calculate blocks on the weighted networks. The algorithm finds the best groupings of insects and plants that maximize the Integrated Completed Likelihood (ICL; Biernacki *et al.* 2000; Daudin *et al.* 2008) of
a model that fits the intensity of interactions between each pair of species as a Poisson draw with a
parameter defined by the blocks each species belong to.

While the LBM approach reveals consistent species groups in complex networks, it neither informs 246 on how species interact within each block, nor does it seek regularities in the arrangement of links 247 within a small group of species. Such information can be obtained by counting the number of motifs 248 observed within networks (Simmons et al., 2019b, 2019a). Motifs are defined by Simmons et al. 249 250 (2019a) as "building blocks" of the network, *i.e.* patterns of possible interactions between a small number of species. If we compare the network to a toy brick house, motifs would be the "building" 251 252 bricks" with different sizes, shapes and colours that can be used to build the house. Motifs contain between two (one pollinator and one plant species) and six species. Motifs do not only consider 253 direct interactions, but they also consider indirect ones, when the impact of one species on another 254 is mediated by one or more intermediary species (Wootton, 2002, 1994). To calculate how 255 frequently different motifs occurred in our networks, we used the function *mcount* implemented in 256 the new package bmotif in R (Simmons et al., 2019b) and normalized these values using the 257 maximum number of times each motif could have occurred given the number of species in the 258 259 network (correction "normalise nodesets").

260 2.7. Insect roles and specialization index

Within motifs, species (nodes) can be found at different positions. Each position reflects a particular ecological role (*e.g.* pollinator species linked to at least two plant species with one of these connected to another pollinator species) and the same species can appear at different positions in different motifs. We calculated the sum-normalised frequencies of each position for each species using the *node_position* function implemented in the *bmotif* package in R (Simmons et al., 2019b).

We also calculated the standardized specialization index d' (Blüthgen et al., 2006), but we did not use the d' values provided by the dfun function in the bipartite package (Dormann et al., 2009) as they sometimes yielded spurious results based on the computation of the minimal d value (*e.g.* reporting low d' for species with only one partner in the network). However, we used the d and dmax values, obtained from the dfun function, and we calculated the d' index, for each plant and insect species, as the ratio of the d-value (Kullback-Leibler divergence between the interactions of the focal species and the interactions predicted by the weight of potential partner species in the overall network) to its corresponding *dmax*-value (maximum *d*-value theoretically possible given
the observed number of interactions in the network).

275 2.8. Comparing network structure and species roles using a null model

276 To understand to what extent the networks obtained using pollinator visit records did not bias the representation of the network when compared to those obtained through pollen identification, we 277 278 compared species roles and specialization (node-level statistics), motif counts and the congruence 279 between latent blocks (network-level statistics) using a null model accounting for the difference in 280 sample size between visit- and pollen-based networks. We thus constructed null model networks (hereafter called "simulated" networks) in which we fixed the number of interactions per pollinator 281 282 species as found in the visit-based network, but with randomized interactions pairs obtained from the interactions recorded in the pollen-based network. In other words, we can consider a simulated 283 284 network as the result of a virtual observer that samples the same insects visiting plants, but the 285 plants on which the insects are virtually observed are drawn from the distribution given by the pollen-based network. We performed 10,000 randomizations using the function *rmultinom* (package 286 287 stat in R) to generate multinomial distribution drawings following the interaction frequencies reported in the pollen-based network. 288

To gauge if the network structure changed between the two networks, we compared the results of 289 290 LBM and motif analyses between visit-based networks and simulated ones. Then, to detect whether species roles changed between networks, we compared results on specialization and node positions. 291 292 Including connectance, H2, d', NMI, motifs and positions, we performed 197 tests in the site of F, 117 tests in the site of CG and 107 tests in the site of R and we adjusted all p-values in each site 293 294 using the function *p.adjust* (package *stat*) and the false discovery rate correction method of Benjamini-Hochberg ("BH" or "fdr", Benjamini and Hochberg, 1995). The numbers of tests are 295 296 different because we had different numbers of species in the three sites.

Latent Block Model – We performed LBM on weighted versions of the visit-based, pollen-based and simulated networks (10,000 simulations). To show the species rearrangement among groups between the visit- and pollen-based networks, we used alluvial diagrams (package *alluvial* in R). In order to assess whether changes in block memberships of species between pollen- and visit-based networks was expected due to changes in sampling intensity, we computed the congruence between the classifications given by node memberships of, first, the visit- and pollen-based networks and, then, the pollen-based network and each of the simulated networks, using the normalized mutual information index (NMI), implemented as method "*nmi*" of the function *compare* in the R package *igraph* (Danon *et al.* 2005; Astegiano *et al.* 2017). NMI values range between 0 (no congruence
between classifications) and 1 (perfect congruence). The distribution of NMIs obtained when
comparing the blocks of the pollen-based networks and those of the simulated networks allowed us
to compute the probability (p-value) that the NMI between the visit-based and pollen-based blocks
was significantly less than expected from the null model. Corrected p-values less than 5% were
deemed significantly inferior to the null model expectation.

Motifs – The motif analysis was performed on binary networks (Simmons et al., 2019b) and explored all motifs with up to 6 species. The frequency of each motif in the visit-based network was compared to the corresponding frequencies in the ensemble of randomized networks using a twotailed test for the purpose of significance (*i.e.* the difference in frequency was deemed significantly different if it fell below 2.5% or above 97.5% of the simulated cumulated frequencies for the same motif).

Positions - To explore if insect and plant species had different roles in the networks based on visits 317 vs. pollen, we calculated the frequency with which species occurred at different positions within all 318 possible motifs of 2 to 6 species. This vector of position frequencies represented the species' "role" 319 in the network. We then calculated the distance of each species' role to the centroid of all the 320 321 simulated roles for the same species, and compared this distance to the distribution of distances between simulated roles and their centroid, with observed distances greater than 95% of the 322 simulated distances deemed as significantly different from the null expectation. To account for 323 heterogeneous variances and correlations between position frequencies (*i.e.* coordinates in species' 324 role vectors), we used Mahalanobis distance on modified coordinates obtained by first running a 325 principal component analysis (PCA) on the set of all roles of all species in all simulated networks. 326 327 The covariance matrix used in the Mahalanobis distance was simply the diagonal matrix of singular values associated with the principal components of the PCA. The modified coordinates of the 328 329 centroid and the observed role of a given species were obtained by projecting their position frequencies into the PCA space. 330

All analyses were performed in R version 3.5.2 (R Core Team, 2018).

332 **3. Results**

333 *3.1. Characterisation of plant-pollinator interactions*

In the month of July, we recorded a total of 96 flowering species, 63 in the site of Fourches (F), 33 in the site of Château Gaillard (CG) and 32 in the site of Riez (R). However, these species were not all visited by pollinators, and those with neither visit nor pollen found on insects (12 species in the site of F, 14 in the site of CG and 16 in the site of R) were not considered in the analysis.

We sampled 574 visiting insects overall, but for the statistical analysis we only used female insects with the information on both types of pollen (collected and scattered). For the following analyses, we used 391 insects overall, 173 in the site of F, 128 in the site of CG and 90 in the site of R.

341 Visit- and pollen-based networks in the same site have comparable number of species (i.e. the 342 number of insect species is fixed, but the number of plant species can vary depending on the sampling, *i.e.* visit or pollen), except in the site of Fourches: for the site of Fourches 50 insect 343 species x 44 potential plant species (29 plant species in the visit-based network vs. 40 species in the 344 pollen-based one); for the site of Château Gaillard 22 insect species x 18 plant species (13 in the 345 visit- and 18 in the pollen-based networks) and for the site of Riez 19 insect species x 16 potential 346 plant species (12 in the visit- and 15 in the pollen-based networks). For three insect species (2 347 species in the site of CG, Lasioglossum laticeps and Lasioglossum politum, and 1 species in the site 348 349 of R, *Halictus rubicundus*) which were sampled once in the visit-based network, we did not record any interaction in the pollen-based network due to the low number of pollen grains (< 5) or to 350 interactions with plant species not included in the botanic inventory (Fig. A.3, A.4). These species 351 352 were thus excluded from the analyses in the problematic sites. In the site of Fourches we recorded 179 visit-based interactions and 340 pollen-based interactions; in the site of Château Gaillard we 353 354 recorded 130 visit-based interactions and 228 pollen-based interactions and in the site of Riez we recorded 93 and 173 interactions in the visit- and the pollen-based networks, respectively. Overall, 355 356 with the pollen information we doubled the number of interactions in all sites.

357 *3.2. Plant-pollinator networks analysis*

When we compared network connectances, *i.e.* the proportion of realized links over all possible ones, we found that the pollen-based network connectance was always higher than the visit-based network connectance in the three sites (Table 1). We observed the opposite pattern for the network specialization index, since the H_2 values were higher in the visit-based network than in the pollenbased one in all sites (Table 1). However, when we compared the visit-based network and the

Analysis	Region	Site	Visit-based network	Pollen- based network	Simulated network	adjusted	p-value
Connectance	Occitanie	Fourches (F)	0.07	0.09	0.07	1	n.s
	Normandie	Château Gaillard (CG)	0.17	0.20	0.14	1	n.s
	Hauts-de-France	Riez (R)	0.18	0.22	0.16	1	n.s
H2	Occitanie	Fourches (F)	0.52	0.33	0.47	0.64	n.s
	Normandie	Château Gaillard (CG)	0.32	0.29	0.37	0.35	n.s
	Hauts-de-France	Riez (R)	0.36	0.30	0.44	0.13	n.s

simulated networks, we did not find any significant difference both for the connectance and the H₂
index (Table 1).

365

Table 1. Results for the analyses of networks connectance and H_2 (network specialisation index) in the visitbased and pollen-based networks and for the simulated networks in the three sites. The adjusted p-values refer to the comparison of statistics between the visit-based network and the simulated networks.

In order to compare network structures between visit- and pollen-based networks, we performed 369 LBMs (Fig. 1) and compared the classification induced by latent blocks using NMI (Table 2). In the 370 site of Fourches, we found a total of 5 blocks (2 insect blocks and 3 plant blocks) in the visit-based 371 network and a total of 7 blocks (4 blocks for insects and 3 for plants) in the pollen-based network. 372 In the site of Château Gaillard, we found 7 blocks (4 for insects and 3 for plants) in both networks, 373 374 and a similar pattern for the site of Riez, but with 5 blocks (2 for insects and 3 for plants) in both networks. Block clustering largely followed species degrees, *i.e.* the number of partners (high, 375 medium and low degree, Fig. A.6). We observed plant species rearrangements in all sites (green 376 lines in the alluvial diagrams), but insect block rearrangements only in the sites of CG (in two insect 377 378 species, Andrena flavipes and Seladonia tumulorum) and R (for one insect species, Lasioglossum pauxillum). Block rearrangements are mainly due to the higher number of links in the pollen-based 379 network but not to substantial changes in the network structure. The higher number of blocks found 380 in the pollen-based network in the site of Fourches (Fig. 1) is due to the occurrence of two new 381 blocks in the group of insects: the first block in the visit-based network (constituted by three species 382 with the highest degrees, Fig. 1 and Fig. A.6 Fourches visits) split in two blocks in the pollen-based 383 network (blocks 1 and 3, Fig. 1 and Fig. A.6 Fourches pollen); and the fourth block in the visit-384 based network (Fig. 1 and Fig. A.6 Fourches visits) also split in two other blocks of species 385 (respectively with species with medium and low degree in the pollen network, Fig. 1 and Fig. A.6 386 Fourches pollen). Even if we found species rearrangements among groups between the visit- and 387 pollen-based networks in all three sites (Fig. 1), the network structures were not intrinsically 388 different. When we compared the congruence between the memberships of species in the visit- and 389

- pollen-based networks using the NMI, we obtained NMI values close to 1 (perfect congruence) in
- all sites (Table 2). Moreover, we did not find any significant difference when we compared these
- 392 NMIs with those obtained from comparisons of the pollen-based network and each of the simulated
- 393 networks.



Figure 1. Alluvial diagrams showing the species rearrangement among blocks between the visit- and pollen-based networks. Green lines show the species rearrangement for plant species and orange and yellow lines for insect species. The plant species that changed modules are *Linum* sp. from block 3 to block 7 (dark green line) and *Lotus delortii*, *Ononis striata* and *Sedum* sp. from block 4 to block 6 (pale green line), in the site of F. In the site of CG the plant species that changed from block 6 to block 7 is *Ononis natrix* and the insects species that changed from block 3 to block 1 are *Andrena flavipes* and *Seladonia tumulorum* (orange line). Plant species that changed block in the pollen-based network, in the site of R, are *Rubus plicatus* and *Trifolium repens* and the insect species that changed from block 2 to block 1 is *Lasioglossum pauxillum* (yellow line).

Site	NMI Visit-based network vs pollen-based network	NMI Pollen-based network vs Simulated networks (quantile)				adjusted p- value	
	_	2.5%	50%	95%	97.5%	_	
Fourches (F)	0.76	0.68	0.76	0.82	0.82	0.98	n.s.
Ch. Gaillard (CG)	0.80	0.76	0.84	0.89	0.90	0.31	n.s.
Riez (R)	0.84	0.73	0.83	0.88	0.94	0.75	n.s.

402

403 Table 2. Normalized mutual information (NMI) values obtained in the three sites when we compared the 404 congruence between the classifications given by node memberships of, first, the visit- and pollen-based 405 networks (NMI visit-based network) and, second, the pollen-based and each of the simulated networks (NMI 406 simulated networks). The p-value corresponds to the probability that the NMI between the visit-based and 407 pollen-based blocks was inferior to what would be expected from the null model.

408 In general, when we compared the network structure using the motifs, we did not find important differences between the visit-based network and the simulated networks. We did not find any 409 410 significant difference when we compared the frequency of each motif in the visit-based network to the corresponding frequencies in the simulated networks in the site of Fourches and Riez. However, 411 we found significant differences for three motifs (motifs 16, 33 and 43; see Fig. 3 in Simmons et al. 412 2019a) in the site of Château Gaillard. All three motifs were less represented in the simulated 413 networks than in the visit-based network (Fig. 2). Motif 16 is constituted by 5 nodes (*i.e.* species) 414 and 6 links, with two species of pollinators (on the top level) and three species of plants (bottom 415 level), while motifs 33 and 43 are constituted by 6 nodes and 7 links, with three pollinators and 416 three plants species in the motif 33 and two pollinators and four plant species in the motif 43. In 417 418 motifs 16 and 43, all species of one group interact with all species in the other group, thus all the possible interactions between the two groups of species are realised. In motif 33 there are two 419 pollinators out of three that are generalist species, *i.e.* they interact with all the plant species, while 420 the third pollinator is a "specialist" species which interacts with only one "generalist" species in the 421 plant group. All the plant species are "generalist" species, but only one plant species interacts with 422 all the partners in the pollinator group, while the other two species only interact with two 423 424 pollinators.



Figure 2. Motifs 16, 33 and 43 in the site of Château Gaillard. Red triangles correspond to the frequency value (corrected value) in the visit-based network and
the boxplot and outliner dots correspond to all the frequency values in the simulated network.

We found that several species in the site of Château Gaillard and Riez had significantly different roles (adjusted p-value < 0.05, Tables A.2, A.3, Fig. 3) when we compared the simulated distances to their visit-based distances, but we did not find any role change at all in the site of Fourches (Table A.1).

In the site of Château Gaillard, we found 13 species significantly more distant from the simulated 433 centroid (seven insect species, Anthidiellum strigatum, Bombus lapidarius (Fig. 3a), Ceratina 434 cucurbitina, Lasioglossum interruptum, Megachile willughbiella, Osmia rufohirta and Trachusa 435 byssina, and six plant species Allium sphaerocephalon, Centaurea scabiosa (Fig. 3b), Echium 436 vulgare, Origanum vulgare, Scabiosa columbaria and Teucrium sp.; Table A.2). In the site of Riez 437 438 we found three species (one insect species, Osmia bicolor (Fig. 3c), and two plant species, Achillea millefolium (Fig. 3d), and Prunella vulgaris) that had significantly different roles between the visit-439 based and the simulated distances (Table A.3). 440

441 We also compared for each species the specialization index d' calculated in the visit-based network 442 to the average d' of the simulated networks. We found that the specialization of most species was 443 not significantly different in the two networks in all sites (Tables A.1, A.2, and A.3). We recorded significant differences in the specialization level for 8 species in the site of F (five insect and three 444 plant species, with two insect and two plant species more specialized in the simulated networks), for 445 two species in the site of CG (one insect and one plant species, both appearing more specialized in 446 the simulated networks) and for five species in the site of R (three insects species out of four more 447 specialized in the simulated networks and one plant species). 448

Overall, nearly half of the species for which we found significant differences in their node positions or/and in the specialization level were singletons (13 species out of 27) in the visit-based or pollenbased network, *i.e.* species that had only one observed interaction (Tables A.1, A.2 and A.3). Only four species out of the 27 (one insect and one plant species both in the sites of CG and R, Tables

453 A.2 and A.3) showed significant differences in both their role and specialization level.



Figure 3. The PCA plot shows the significant distance (adjusted p-value < 0.05) along principal axes 1 and 2, between the visit-based position (red triangle), the
 simulated centroid (black triangle) and the convex hull (black lines and dots) obtained on the 95% of the simulated positions which were close to the centroid
 (grey dots) in the randomized network, in four examples of species among the 13 species that showed significant differences:(a) *Bombus lapidarius* and (b)
 Centaurea scabiosa in the site of Château Gaillard (Normandie) and in (c) *Osmia ruforhirta* and (d) *Achillea millefolium* in the site of Riez (Hauts-de-France).
 The visit-based distance was greater than 95% of all the simulated distances. Photo credits: Atlas Hymenoptera and Acta plantarum.

460 **4. Discussion**

Plant-pollinator networks are mainly constructed using direct observations of plant-pollinator 461 interactions in the field, a method subject to undersampling (Blüthgen, 2010; Olesen et al., 2011; 462 463 Vázquez et al., 2009). The problem of undersampling is much higher in richer communities where some flower visits are scarcer and hence more difficult to detect (Sørensen et al., 2011). The use of 464 pollen found on insect bodies is an alternative method that might help reconstruct the insect 465 visitation history and give a better image of the whole network. Few studies have compared the 466 467 visit- and pollen-based networks (Bosch et al., 2009; Pornon et al., 2017), and all of these 468 comparisons have used classic networks metrics, which are known to be influenced by network 469 dimensions (Astegiano et al., 2015; Blüthgen et al., 2008; Staniczenko et al., 2013).

In our study, we compared plant-pollinator networks constructed (i) from direct observation of pollinator visits and (ii) from identification of pollen found on these same insects in three different calcareous grasslands. The three plant-pollinator networks used in this study showed differences in the identity and number of species in both plants and insects. We used a null model approach (*i.e.* simulated networks), accounting for differences in network size, to understand how differences in sampling method, not intensity, can contribute to changes in observed network structure.

476 As expected (Bosch et al., 2009), our results show that pollen identification increases the number of 477 observed links and always yields richer and more connected networks (Table 1), independently from the site richness and diversity, since in all the sites we doubled the number of links when using 478 the pollen information. Nevertheless, the pollen-based links often confirm the links observed in the 479 480 field (Alarcón, 2010; Popic et al., 2013). We did not find any significant change in any of the study sites when we compared network structures between visit-based and simulated networks. This 481 482 finding, which holds for all three sites, partially invalidates the hypothesis that richer communities (here, the site of Fourches) would lead to more pronounced differences between networks obtained 483 by the two methods. However, we found changes in the species roles for some insect and plant 484 485 species.

Although we observed that the use of pollen data increased the number of interactions (we doubled the number of interactions in all sites), pollen information mostly increased the number of links for abundant and already highly connected species (number of links > 5 in the visit-based networks, Tables A.1, A.2 and A.3), while for rare (singletons) and not abundant species we recorded few interactions even in the pollen-based network. Since visit-based network construction is essentially pollinator-based (and not plant-based), the information given by pollen found on insects is especially useful to add links to plant species that were not observed in the visit-based network

(plant species with no links in the visit-based network, Tables A.1, A.2 and A.3). Indeed, block
rearrangements are observed more often in plants than in insects (Fig. 1 and A.6). However, block
changes in the LBM representation did not correspond to changes in species position.

Block rearrangements are influenced by the number of links and the species degree, *i.e.* the number 496 497 of partners with which a species interacts, but it neither informs on species role nor specialization. For example, the singleton species Prunella vulgaris in the site of Riez clustered in the same block 498 (block 4) in both visit- and pollen-based networks (Fig. 1). Nevertheless, it was the only plant 499 species for which we observed a significant change in its role and specialization degree in this site. 500 In the visit-based network, this species was found in interaction with only one insect species (Fig. 501 502 A.6, Riez visits), *Ceratina cyanea*, in a one-to-one interaction ("direct interaction"), and thus only 503 in position 1 (in motif 1). Conversely, in the pollen-based network, even if P. vulgaris always interacted only with C. cyanea (Fig. A.6 Riez pollen), C. cyanea interacted with two new plant 504 505 species (Trifolium repens and Centaurium erythraea). Thus, the specialization for C. cyanea changed significantly. Moreover, the specialization level and the role of P. vulgaris also changed 506 507 significantly since in the pollen-based network its interaction with C. cyanea was affected indirectly by two other plant species, which may be potential competitors. Moreover, all the new positions of 508 P. vulgaris in the pollen-based network, were "unique" in more complex motifs, i.e. P. vulgaris 509 interacted with one generalist insect species that had other interactions with other plants (Simmons 510 et al., 2019a). Similarly to P. vulgaris, Achillea millefolium, in the same site, was a singleton in the 511 512 visit-based network while it gains one link in the pollen-based network. This new interaction was observed with Lasioglossum pauxillum which was a "super-generalist" species (visiting 11 plant 513 species). Therefore, the role of A. millefolium changed significantly (Fig. 3d) through possible 514 indirect interactions with new potential competitors. These examples show that indirect interactions, 515 516 *i.e.* the impact of one species on another mediated by other intermediary species, are important to give a more complete picture of the species' role when comparing networks, especially when 517 518 accounting for singleton species in the visit-based network.

We also found changes in species roles for 6 species which were more connected in the visit-based network, *i.e.* with more than 5 observed interactions, such as *Bombus lapidarius* and *Centaurea scabiosa* in the site of Château Gaillard (with 20 and 8 interactions, respectively; Fig. 3a, b, Table A.2). In "complex" motifs where all species are generalists in both groups and all interact together, changes in species roles through indirect interactions are expected to be stronger than in "simple" motifs which are composed of specialist species that affect each other indirectly via their effect on one generalist species (Simmons et al., 2019a).

In most species for which we observed a significant change in their positions or specialisation 526 degree, we recorded a slightly higher number of links or the same number of links in the pollen-527 based networks than in the visit-based ones, and only 3 species out of 27 nearly doubled their 528 interactions (Tables A.1, A.2 and A.3). However, for four species, *Anthyllis vulneraria* in the site of 529 530 F, Origanum vulgare and Centaurea scabiosa in the site of CG and Lasioglossum fulvicorne in the site of R (Tables A.1, A.2 and A.3), we recorded a lower number of links in the pollen-based 531 532 network than in the visit-based one, since some interactions were only observed in the field but they 533 were not confirmed with pollen identification (blue squares in the Fig. A.2, A.3 and A.4), which might explain the significant difference in their specialisation level or species role obtained when 534 we compared the visit-based network to the simulated networks. For both A. vulneraria and L. 535 536 fulvicorne, the specialization level recorded in the visit-based network was always lower than the one recorded in the simulated networks, which means that both species were less specialized (in the 537 538 visit-based network) than expected if the interactions had been drawn out of the ones recorded by pollen grains. For C. scabiosa the number of links recorded in the pollen-based network was lower 539 540 than in the visit-based one since the interactions with two insect species, Bombus lapidarius and Osmia leaiana, were not confirmed by pollen identification (Fig. A.3), probably because the two 541 visitors were not carrying enough pollen grains (less than 5) of this plant species. Therefore, in the 542 pollen-based network the loss of partners and their interactions influenced C. scabiosa's role, 543 especially in highly connected motifs (*i.e.* motifs where all species in one group interact with all 544 species in the other group) such as motifs 16 and 43, which were less represented in the simulated 545 networks than in the visit-based network. Consequently, the loss of the interaction with C. scabiosa 546 indirectly induced a change in the position of *B. lapidarius* (Fig. 3). 547

Since the focus of our study was to compare representations of networks borne out of two different 548 549 methods (observation of pollen vs. observation of visits), but taken at equal sampling intensity, our results do not comprise the obvious differences seen in raw pollen vs. visit comparisons, i.e. that 550 551 more detailed approaches (such as pollen-based network building). We did not find any significant changes in network structures once the intensity of sampling was taken into account, but we 552 553 observed important changes at the species level in all the three sites. Indeed, we found differences both in species role and specialization in a few species, as also evidenced by other studies 554 (Ballantyne et al., 2015; Lucas et al., 2018b). We showed that non-significant change in network 555 556 structure can mask more subtle changes of species roles and specialisation level. However, these 557 changes are in part observed in singleton species such as P. vulgaris in the site of Riez, which 558 showed a low number of links both in the visit- and in the pollen-based networks. Singleton species 559 are expected even in well-sampled communities, since they are often considered as rare species

accounting for rare interactions (Bascompte and Jordano, 2013; Novotný and Basset, 2000).
Moreover, in our study we focused on wild bee species, but pollination networks are also composed
of other pollinator species (Bosch et al., 2009; Lucas et al., 2018b; Pornon et al., 2016). Hoverflies,
beetles, butterflies and moths, and ants can carry less important amount of pollen grains than bees
(Alarcón, 2010), due to their low hairiness, but can nonetheless influence the network structure and
the comparison between visit-based and pollen-based networks.

566 To conclude, our results suggest that more detailed sampling, obtained from pollen found on insect bodies, does not lead to the construction of an intrinsically different network, independently from 567 the site richness and diversity. Almost all of the significant differences are found at the species 568 569 level, not at the network structure level, with singleton species accounting for half of these species-570 level differences. Overall, this suggests that recording plant-pollinator interactions from pollinator visit observation is enough to provide a satisfactory representation of the network structure. 571 However, the use of pollen can provide a more exhaustive image at the species level, highlighting 572 important changes in species role and specialization, especially for studies investigating pollinator 573 574 effectiveness and/or dealing with scarce pollinators. Since pollen identification is a time-consuming endeavour, new methods such as DNA-barcoding might simplify and accelerate pollen 575 identification in the future if improved with new specific (regional or local) botanic databases. 576

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578 Author contributions: NDM and FM conceived the project. NDM, NH, YP, BS and MZ 579 conducted the fieldwork and provided the data. MZ and NMD prepared the insect slides and ES 580 prepared the Pollen Atlas. PM provided part of the pollen identification and conducted the 581 preliminary analyses. NDM conducted the analysis and prepared the manuscript. CM helped with 582 analyses. FM supervised the analysis and edited the manuscript. NH, YP and BS contributed to all 583 later versions.

584

585 Acknowledgements

586 Financial support was provided by the ANR projects ARSENIC (grant no. 14-CE02-0012) and 587 NGB (grant no. 17-CE32-0011), the Region Nord-Pas-de-Calais and the CNRS. We also thank 588 David Genoud, Matthieu Aubert, Denis Michez, Michael Terzo and Alan Pauly for insect 589 identification and all the students who took part in the field campaign and in the laboratory analysis. 590 We are grateful to Sophie Donnet and Sarah Ouadah for providing us with the R script for drawing alluvial diagrams. This work is a contribution to the CPER research project CLIMIBIO. The authors
thank the French Ministère de l'Enseignement Supérieur et de la Recherche, the Hauts-de-France
Region and the European Funds for Regional Economical Development for their financial support.
We thank the editor (Isabelle Dajoz) and two anonymous reviewers for insightful comments on the
manuscript.

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