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**Designing sampling protocols for plant-pollinator interactions - timing, meteorology, floral resources assessment frequency and failed captures matter**

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## **Abstract**

Plant-pollinator interactions are key components of ecosystem functioning and are thus increasingly studied. Among all approaches used to estimate these interactions, pollinator capture along transects is a broadly used and recognized method. However, the specific choices of the sampling design can strongly influence observations of insect visits, and bias ecological interpretations. Yet, there is no agreement on the best transect design. The sampling intensity (number and length of transects) is an important component of those choices but not the only one. Here we propose to investigate the influence of three other facets of protocol choices that commonly arise when designing pollination transects: the influence of sampling conditions that can interact with ecological variables of interest or bias the observations, the measurement of floral availability frequency and the gestion of observed but non captured insectes. We quantified the importance of those three protocol choices using a large dataset of 720 plant-pollinator transects in protected wet meadows in France. Our results demonstrate the necessity of (i) covering large ranges of temporal and meteorological conditions for each site, and (ii) repeating the assessment of patch attractiveness for pollinators (a major covariate, usually simply derived from one-off vegetation surveys). Additionally, we show that (iii) for analyses of visitation density across insect groups, failed insect captures should not be dismissed but be well integrated into the analyses. Overall, this research identifies three key choices in transect design and highlights how they can influence our understanding of plant-pollinator interactions.

**Keywords:** meteorological conditions, patch attractiveness, failed captures, pollination, insect sampling, flower visitation, transect, phenology

**Running head:** Designing plant-pollinator transects



## Introduction

Pollination is a key component of ecosystem functioning as it influences vegetation diversity and dynamics (Benadi et al. 2013; Lundgren et al. 2016) and is crucial to agricultural activities (Aizen et al. 2009; Reilly et al. 2020). It is highly sensitive to global changes as climate variations can induce phenological mismatches between plants and pollinators (Hegland et al. 2009), and land use modifications or biotic invasions can affect the availability of floral resources, pollinator communities and pollinator behaviour (Morales & Traveset 2009). It is thus crucial to understand the mechanisms underlying plant-pollinator interactions and their response to global changes. Doing so requires sampling and analysing pollination in varying environmental contexts. Two types of methods have been proposed to estimate pollinator visits on flowers (e.g, random walk, fixed observation points), and transects of direct observations have been shown to provide the most cost-effective information on pollination networks (Gibson et al. 2011).

For each approach, the sampling design can strongly influence the observations of plant-pollinator interactions observations. The sampling intensity (e.g, number and length of transects), for instance, is largely informed in pollination networks studies (Hegland et al. 2010; Chacoff et al. 2012; Falcão et al. 2016). However, a number of environmental factors can also influence the observations, and neglecting them may generate biases that alter ecological interpretations. The date and time of each sampling event (as indicators for plant phenology and insect behaviour) as well as meteorological conditions are known to have a huge impact on the abundance and identity of observed visits (Bergman et al. 1996; CaraDonna et al. 2017; Goodwin et al. 2021). Some insects are more active during certain times of day (e.g, Syrphidae are mostly observed during morning or late hours) and certain periods of the year (e.g, Coleoptera are active earlier in the year than *Lepidoptera* (Duchenne et al. 2020)). Variations in meteorological conditions affect some insect taxa more than others (e.g, Diptera are less tolerant to windy conditions than Hymenoptera). Additionally, patch attractiveness, usually estimated by the area occupied by open flowers, has been shown to be an important predictor of pollination activity (Wood et al. 2015; Templ et al. 2019), but how frequently

this factor should be recorded within the season remains unknown (e.g, during each transect vs. once per season). Another frequent concern in the field is how to handle observed visits when the insect has escaped and can only be identified at the order level. This is particularly important when the study covers multiple groups of insects with various escape strategies (e.g, falling down, flying away quickly, burying themselves into the flower).

In practice, time and cost constraints are driving protocol choices. These constraints typically impact the effort invested into repeating the sampling at different times and days, but also under various meteorological conditions, estimating patch attractiveness through frequent flower area measurements (counting flowers can sometimes take longer than running a transect), or tracking failed insect captures. However, typical research questions usually aim at identifying the ecological variables that are driving plant-pollinator interactions, independently of the specific protocol choices. Yet, there has been no evaluation of the noise or statistical bias that can arise from those sometimes neglected choices, and how they can propagate onto our ecological interpretations.

Here we propose to investigate the influence of protocol choices using a large the data from an intensive survey of insect visits in wet meadows of two protected areas in the French Alps. We answered three key questions that commonly arise when designing pollination transects:the ecological variables of interest (1) To what extent can the timing and the meteorological conditions of the sampling events blur the effects of the ecological drivers of interest? (2) How frequently should the flower ressources across the transect be recorded (once per season vs. before each transect observation)? (3)Is it important to keep track of the visits when the insect escapes and is not identified?

)

## **Materials & Methods**

### ***Study system***

We surveyed the composition of plants and insects along a gradient of *S. canadensis* density in two protected areas of the French Alps (Figure S1). *S. canadensis* is an Asteraceae from North America introduced in Europe as an ornamental species during the 1900s that has become one of the most invasive plants in European and Asian wetlands (Weber 1998; Moroń et al. 2009). *S. canadensis* can affect both: (i) the native vegetation (in terms of composition, phenology and resources allocation) and (ii) insect behaviour (attracting insects with a high production of pollen and nectar when blooming). We thus expected a negative effect of *S. canadensis* on insect visits before and during its flowering time through a reduction of the native floral resources (or at least its visibility) and an attraction of insects to this resource at the expense of native flora pollination. We selected six wet meadows with a homogeneous management regime and type of vegetation. Each of them presented a broad variation in *S. canadensis* density (0 to 117 stems per m<sup>2</sup>) defined as our focal ecological gradient of interest. We laid two to four plots of 100m<sup>2</sup> per meadow (depending on the meadow size) along this invasion gradient (Figure S1).

### ***Transect design and sampling***

We visited each plot 12 to 13 times from mid-May to mid-August, and three observers recorded simultaneously the visits of insects on flowers across three parallel 10m x 2m transects. We sampled only when the meteorological conditions were favourable (temperature over 15°C, wind below 13km/h and cloud coverage below 70%). The observers walked the transects in a 10minute round trip and caught all insects in contact with stamens of entomophilous species (timekeeping was paused during insect captures; building on the two methods presented by Gibson et al. 2011). We captured all insects, regardless to their taxonomic group because there is growing evidence that pollination is performed by other taxa than just Apidae and Syrphidae (Szymank et al. 2008; Orford et al. 2015). We made an exception for western honeybees (*Apis mellifera*) that were easily recognizable and too abundant to be all captured in some plots. All the insects we observed but failed to catch were described by their taxonomic group (western honeybee, wild Hymenoptera,

Diptera, Coleoptera, Hemiptera, Orthoptera, or Lepidoptera) or recorded as “other” if identification was not possible. We kept track of the plant species that was visited by each captured or recorded insect.

Before each sampling event on a transect, we estimated the transect attractiveness via the surface occupied by open inflorescences. We counted the number of open inflorescences for each plant species and multiplied it by a visual estimate of its surface (Table S1). We then obtained an estimation of the total surface of native flowers and the surface of invader flowers. We also recorded the time (*hour*, corrected for day length variations and centered on 12:00am) and the day (*day*) of the sampling event to account for differences in species phenology and behavior across time (CaraDonna et al. 2017). Finally, we measured meteorological conditions: *temperature*, wind velocity (*wind*) and cloud coverage (*cloud*). All these variables were correlated at less than 48%.

### ***Statistical analyses***

We examined the relative contribution of the ecological drivers of interest, and sampling conditions (meteorological conditions, sampling date and time), when explaining pollination activity. In this study, we estimated pollination activity through the number of insect visits per unit of native flowers area (called *visitation density*). We expected that different taxonomic groups would respond differently to *S. canadensis* density.

We thus conducted all analyses on the complete dataset (all observations) but also on eight subsets of that dataset. Each subset corresponded to either (i) the visits provided by one of the five insect groups that were the most abundant in our study area (honeybees, wild Hymenoptera, Diptera, Coleoptera, and Hemiptera), or (ii) the visits received by one of the three dominant entomophilous native plant families (native Asteraceae, Apiaceae, and Lamiaceae).

honeybeewild HymenoWe analyzed these measures of visitation density independently at two time periods: (i) before the flowering of *S. canadensis*, when the invader mostly impacts visitation

density through competition with native plant that can impact the plant community composition, flowers production and visibility (Fenesi et al. 2015), and (ii) during the flowering of *S. canadensis*, when the invader additionally provides insects with important floral resources that can lead to a modification of insect community composition and behaviour (Sun et al. 2013).

We expected *visitation density* to be determined by three ecological drivers of interest: (1) the focal environmental gradient decomposed into the density of *S. canadensis* (*Invader density*) and the surface occupied by its flowers (*Invader flowers area*), (2) the availability of native floral resources (*Native flowers area*), and (3) the local specificities of each meadow in terms of soil, and surrounding landscape configuration (*meadow ID*). In this regard, meadow ID is treated as a fixed effect and not a random effect in this model. In addition, *visitation density* could be influenced by other ecological factors linked to sampling conditions, which can blur the effect of the drivers of interest: (4) phenological variations within (*hour*) and across (*date*) days for plants and insects, and (5) meteorological conditions during observation (*temperature, wind, cloud*).

### **1. Phenology and meteorology**

To quantify the relative importance of phenology and meteorological conditions compared to the ecological drivers of interest on *visitation density*, we used boosted regression trees algorithms (BRT; Elith et al. 2008) A BRT is a recognized form of machine learning technique that assembles (via boosting) multiple small regression trees into a powerful predictive model, and has the advantages of allowing flexible response shapes and interactions among many predictors without losing too many degrees of freedom due to internal regularization. The models were built to predict pollinator *visitation density* from nine explanatory variables including four ecological drivers of interest: *invader density, invader flowers area, native flowers area, meadow ID*, and five sampling condition variables: *day, hour, temperature, wind* and *cloud*. We performed a BRT for nine measures of *visitation density* (all visits, visits per insect type and per plant families) before and during *S. canadensis* flowering. The results of these 18 models are the “reference regressions” for

all subsequent analyses as they correspond to the most accurate analysis possible with our dataset. For each of these models, we estimated their global goodness-of-fit (model  $R^2$ ) and the relative contribution of each variable (hereafter contribution).

## **2. Frequency of flowers area measurement**

In order to check if the frequency of floral area measurement affected the precision of the analyses, we downgraded our data by redefining the *native flowers area* of each plot as its value measured at mid-period (respectively late June and early august). We obtained a dataframe that correspond to the one we would have had if we had measured the flower area only once per period.

We expected the number of visits received per flowers area to be highly sensitive to the patch attractiveness (estimated by *native flowers area*). However, quantifying this area at each sampling event is time consuming and doing it once per period significantly fastens protocols. Within each period (before and during *S. canadensis* flowering), we redefined The response variable, *visitation density* was also modified as it is measured per unit of *native flower area*. We then performed the 18 regressions on this new dataset and compared the  $R^2$  and the contribution of the ecological drivers of interest obtained with those of the reference regressions.

## **3. Influence of observed but non-captured visits**

The number of insects we observed but escaped may varied among observers and may be linked to *visitation density* as the more insects there are in the observed area, the more difficult it is to catch them all. To quantify this potential bias on the results we excluded the non captured visits from our dataset and ran the 18 regressions on this new dataset. Then we compared the model  $R^2$  and contribution of the ecological drivers of interest obtained with these of the reference regressions.

All statistical analyses were conducted in R v.3.6.3 (R Core Team 2020) with the packages `gbm` to run the models and `dismo` to determine the number of trees to include in the models (Brandon Greenwell et al. 2020; Robert J. Hijmans et al. 2020).

## Results

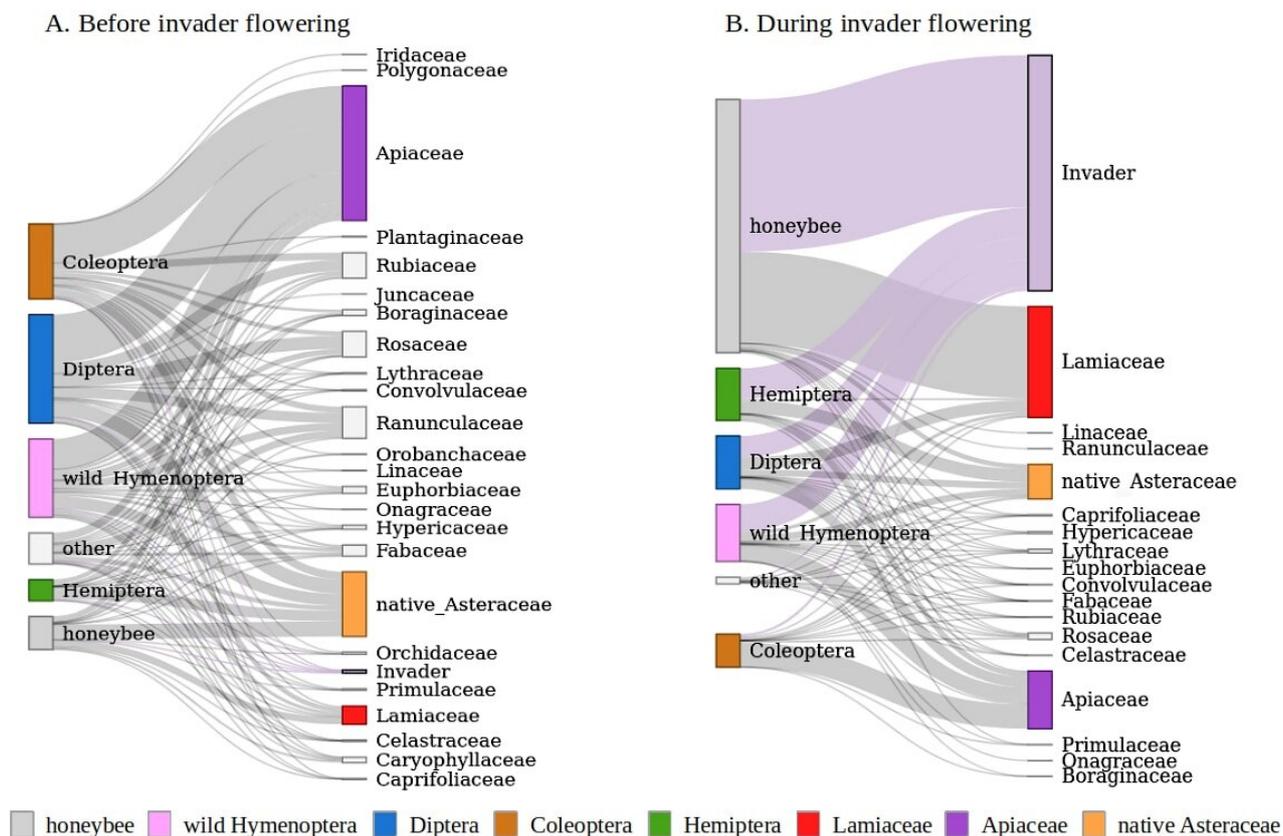


Figure 1. Representation of the relative number of insects captured per flowers before (A) and during (B) invader flowering. Insect species are grouped by taxonomic order (and separating honeybees), and plants species by families (with an extra group for the invasive species). The size of the boxes represents the number of visits given or received by the group.

Overall, we recorded 11,132 insect visits on the 69 native plant species flowering during our study and 6,214 visits on the invader (*S. canadensis*), distributed across 720 transects (60 transects visited 10-13 times each). Apart from visits on the invader (not included in the analyses), most

insect visits (79%) were made to three native plant families: Apiaceae (29%), Lamiaceae (28%), and Asteraceae other than *S. canadensis* (23%). Each plant family attracted different types of insects: Lamiaceae were mostly visited by honeybees (80% of the visits on Lamiaceae), Apiaceae by Coleoptera (35% of the visits on Apiaceae), wild Hymenoptera (25%), and Diptera (17%), and native Asteraceae were visited evenly by all insects groups. Each insect group showed specific preferences: honeybees (28% of all visits) mainly visited Lamiaceae (81% of honeybees visits), Coleoptera (17% of all visits) primarily visited *Apiaceae* (59% of Coleoptera visits), while Diptera (25% of all visits) and wild Hymenoptera (22% of all visits) visited more evenly the different plant families (Figure 1).

### 1. Phenology and meteorology

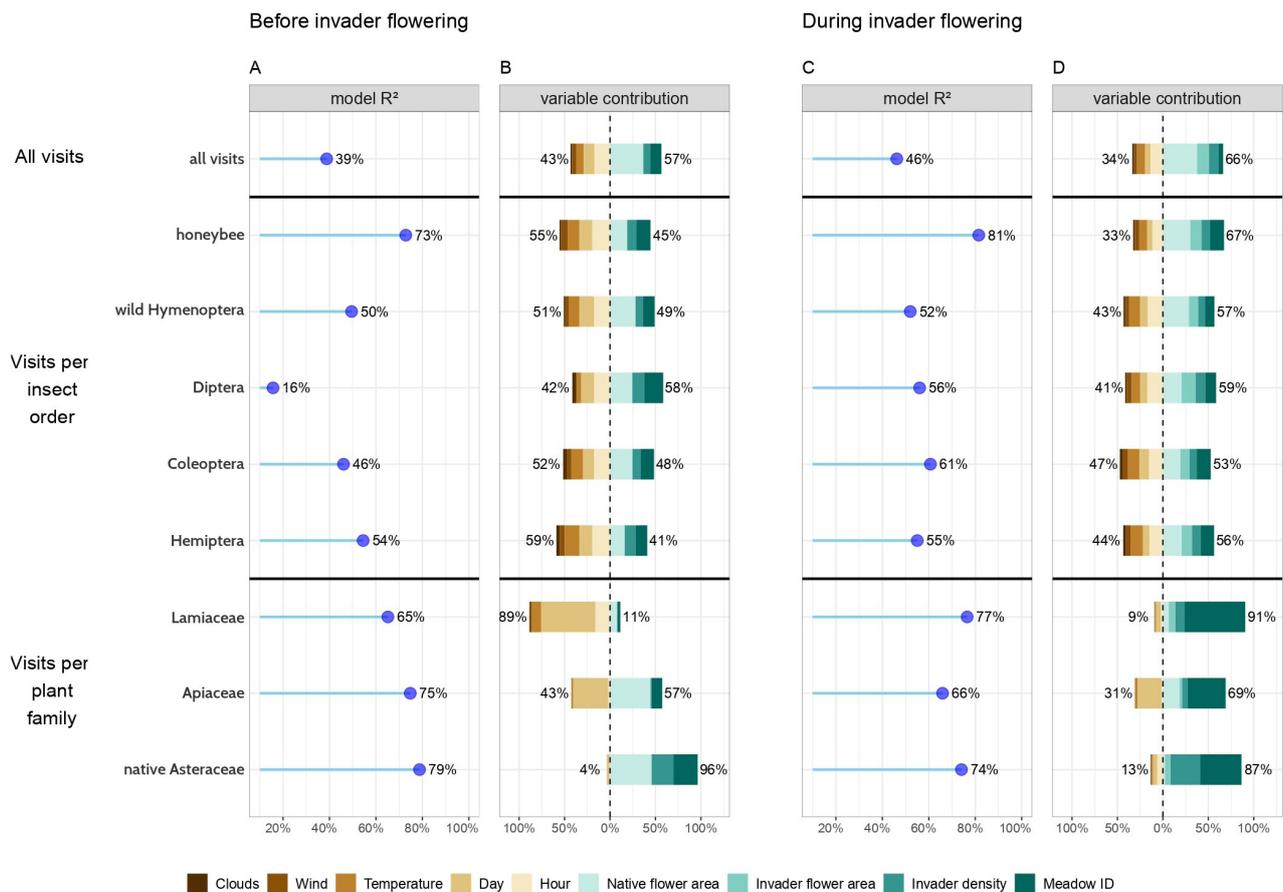


Figure 2. Model performance and variable contribution for the 18 reference models. Model performance is quantified by its proportion of variance explained: R<sup>2</sup> (A & C), and variable

*relative contribution distinguishes between the ecological variables of interest (B & D, green gradient) vs. the variables linked to protocol choices (B & D, brown gradient).*

The goodness of fit ( $R^2$ ) of the 18 models ranged from 39% to 81%, except for the visits of Diptera before invader flowering (16%; Figure 2A,C). The ecological variables of interest contributed more to the model (53%-91%) than sampling date, hour and meteorological variables during invader flowering, but we found the reverse before invader flowering for Lamiaceae and insect groups other than Diptera. The contribution of the variables was similar across taxonomic groups and periods when considering all visits or visits per insect group, but varied a lot across plant families within and between periods (Figure 2B,D). *Native flowers area* was the variable that better explained visitation density in all models (19-46% of the explained variance), except for Lamiaceae, Hemiptera and honeybees before invader flowering, and plant families during invader flowering. *Meadow ID* was the main predictor of visits per plant family during invader flowering (42-67% of contribution) and contributed up to 27% in other models. The *invader density* contributed well in models for native Asteraceae (24%-32%; the group of the invader) but only up to 13% in other models. *Invader flowers area* contributed less than 15% in all models.

The sampling time (*day* and *hour*) largely contributed to most models (17%-41%), except for native Asteraceae and Lamiaceae during invader flowering (3% to 10%). *Temperature* had a moderate contribution to most models (8%-16%; except for Diptera before invader flowering and plant family models). *Wind* and *cloud* contributed to less than 8% in all models (Table S2). However, those three weather variables altogether significantly contributed to most models (14%-25% except for Diptera before invader flowering and plant family models).

## **2. Frequency of floral area measurement**

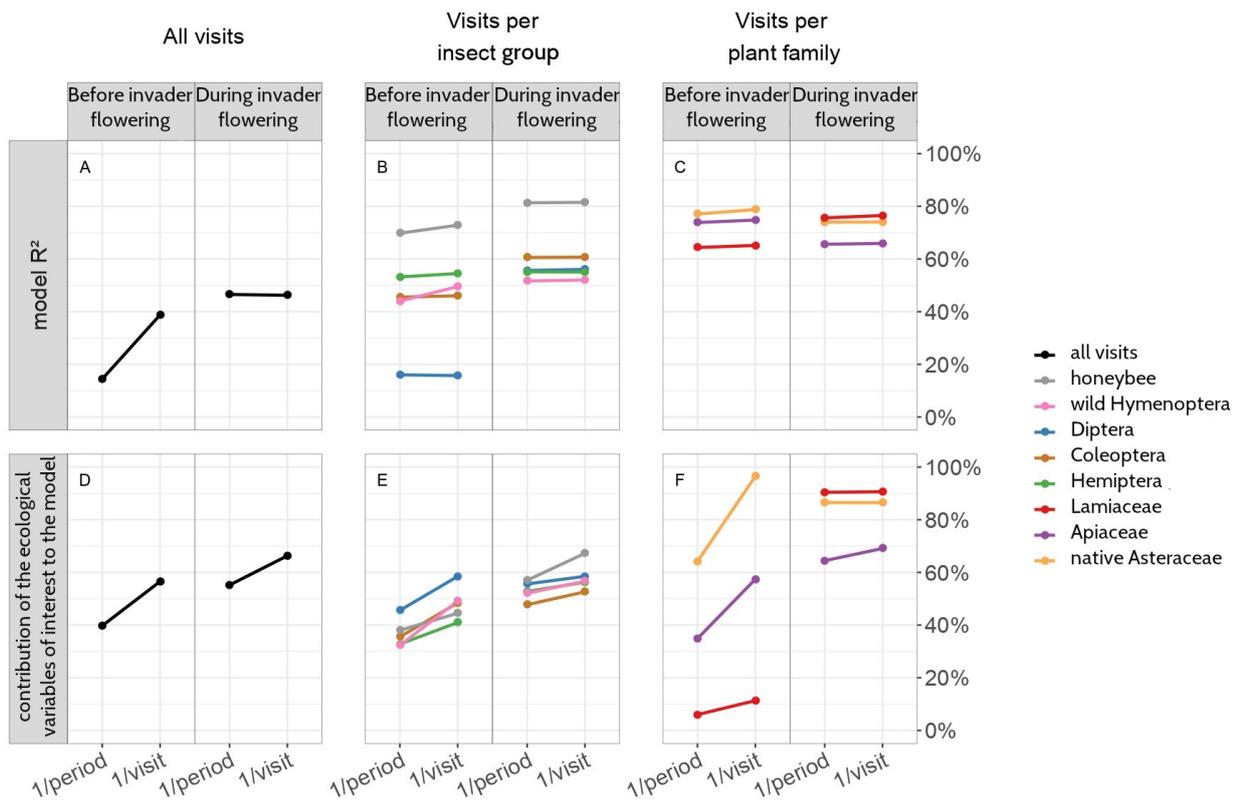


Figure 3. Impact of the frequency of flower area measurement (once per period or once per sampling event) on the model performance ( $R^2$ ; A-C), and on the contribution of the ecological variables of interest (D-F), before and during invader flowering for all groups (A & D), per insect group (B & E), and per plant families (C & F).

The frequency at which flowers area was measured only marginally affected the models in terms of overall performance: reducing this frequency from once per visit to once per flowering period led to a maximum of 5% decrease in  $R^2$ , except when considering all groups before invader flowering (24% decrease in  $R^2$ ) (Figure 3). However, the total contribution of the ecological variables of interest (*native flowers area, invader flowers area, invader density and meadow ID*) was largely reduced before invader flowering (5% to 32% decrease) and during invader flowering (0 to 11% decrease).

### 3. Influence of observed but non-captured visits

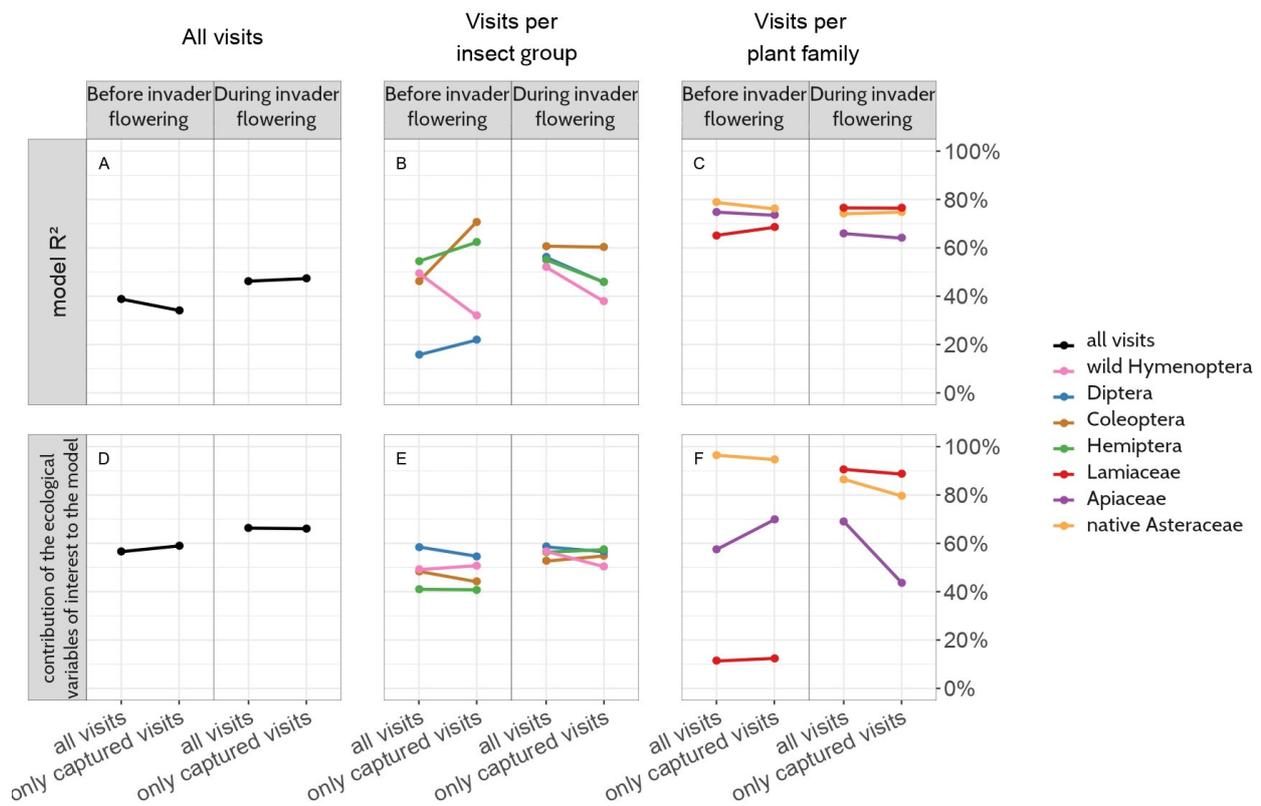


Figure 4. Impact of ignoring observed but non-captured visits on model performance ( $R^2$ ; A-C), and on the accumulated contribution of the ecological variables of interest (D-F). The results are presented before and during invader flowering for all groups (A & D), per insect group (B & E), and per plant families (C & F).

Accounting for observed but non captured visits in the analysis had only marginal effects when considering all species together or honeybees (Figure 4A,D). However, in analyses specific to insect orders, accounting for all visits highly influenced model performance, with various effects across groups but it generally decreased model fit before invader flowering and increased it during invader flowering (by 5% to 24%; Figure 4B). Analyses specific to plant families showed that accounting for all visits only marginally influenced both model performance (less than 3% decrease in  $R^2$ ) and contribution of the ecological variables of interest, except when considering Apiaceae

(12% decrease in contribution before invader flowering and 25% increase during invader flowering; Figure 4C,F).

## **Discussion**

Our results show that in general, the visitation density was highly influenced by: the ecological variables of interest (mainly native and invader flowers areas), phenology and meteorological variables. The contribution of these different variables were consistent across insect groups, but varied a lot across plant groups. We additionally showed that, depending on the focal taxonomic group, models could be largely improved by the use of high quality measures of patch attractiveness (i.e., the frequency of floral area measurement) and the inclusion of escaped insects in the dataset.

### ***1. Phenology and meteorology need to be accounted for in protocols***

With the complete dataset (all captured and non-captured interactions, flower area measured at each sampling event), pollinator visitation density was well predicted by all predictors both before and during invader flowering. The ecological variables of interest, especially the *native flowers area* in the transect, explained most of insect visitation density. This variable is a component of the patch attractiveness (Conner and Rush 1996; Lázaro and Totland 2010) and thus expected to influence insect visits (Templ et al. 2019). Apart from the ecological variables of interest, sampling conditions in terms of phenology (both *day* and *hour*) and meteorological conditions (especially *temperature*) did also strongly influence *visitation density* before and during the invader flowering.

The results differed across insect groups and plant families. Visitation density was better explained for smaller taxonomic units (i.e., single insect order or plant family), than when considering all visits, except for Diptera. This result supports the expectation that insect foraging behavior is generally more similar within than across orders (Romero et al. 2011). Additionally, the relative

contribution of phenological and meteorological variables remained high but differed widely across taxonomic groups. Compared to the global models, the contribution of sampling time and meteorological variables increased for most insect orders, but decreased for most plant families (except for Lamiaceae before invader flowering). This result can be explained by the strong influence of sampling time and meteorological conditions on insect foraging behaviours (compared to plants), and the large range of pollinators interacting with a given plant family. This result highlights the fact that both sampling time (*hour* and *day*) and meteorological conditions (especially *temperature*) can be important to consider when designing the transect protocol, particularly so when investigating insect visits from different insect orders as they can respond differently to the sampling time and meteorological conditions. To do so, transects should be repeated multiple times during the sampling season, at different times of the day, with a large range of meteorological conditions (within suitable conditions for insects to fly). We found only marginal effects of *wind* and *cloud*, but this may be due to the relatively moderate ranges of conditions experienced during the sampling (e.g. up to 70% cloud coverage, and 13 km/h wind).

## ***2. Floral areas should be frequently assessed***

Estimating the amount of floral resources per plant family before a sampling event is crucial because it informs on both the density of insect visits per flower unit, and the attractiveness of the site for prospecting pollinators. It is however a tedious task and it is thus relevant to ask whether reducing the number of assessments of this resource affects the quality of this information. Here we showed that reducing the number of floral surveys from once per sampling event to once per sampling period can affect both the performance of the models (up to 22% reduction of  $R^2$  for global models), and the relative contribution of the ecological variables of interest. We observed that the contribution of these ecological predictors decreases (greatest effect for native Asteraceae and Lamiaceae before invader flowering), while the goodness of fit of the model remains unchanged. This suggests that the influence of sampling conditions can increase and bias our

understanding of the visitation density drivers. The attractiveness of specific flower types is known to be influenced by the surrounding floral resources (Morales & Traveset 2009) that vary a lot across time (mostly before invader flowering). This result brings to attention the importance of floral surveys, and most importantly, their frequency during the sampling period.

### ***3. Non-captured visits mostly influence analyses at insect order level***

It is impossible to reach a 100% catch score when catching insects on flowers. Many insects have escape strategies that make them hard to capture: falling like stones, flying away, digging themselves into the flower, etc. But whether ignoring these non-captured visits strongly biases the results remained largely unexplored. Here we show, in the context of our case study, that ignoring non-captured visits has a very moderate effect when considering either the full set of species, or when analysing visitation density at the plant family level. However, when analysing visitation density for specific insect orders, uncaught insects can influence model performance up to 20% (positively or negatively, depending on the insect group and period). This result is consistent with the fact that escape strategies can be shared by species belonging to the same insect order. It is reassuring that for all visits the influence of non-captured visits remains marginal. This result should be transferred to other case studies with caution, as inevitably the proportion of failed captures will differ across: (i) observers depending on their ability to anticipate the escape strategies, and (ii) across studies depending on the insect species pools, abundance and escape strategies.

## **Conclusion**

When studying the impact of protocols on pollination networks characterisation, most studies focus on sampling intensity and transect design. Our results highlight the need for (i) covering large ranges of temporal and meteorological conditions for each site, and (ii) repeating the assessment of patch attractiveness for pollinators (a major covariate, usually derived from once-off vegetation

surveys). Moreover, we show that (iii) for analyses of visitation density across insect groups, failed insect captures should not be neglected. We hope that this research, on three key choices and how they can influence our understanding of plant-pollinator interactions, will open the path for further investigations on how protocol choices influence our understanding of ecological processes, in other ecosystems and sampling approaches.

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## **Author contributions**

MCG, LG and FM conceived the study. All authors contributed to the field work. JR created the database, MCG performed the statistical analyses, and MG helped with the figures. MCG wrote the first draft of the manuscript, which was significantly improved by all authors.

## **Data availability statement**

Raw data and derived data supporting the findings of this study are available from the corresponding author MCG on request.

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### **Figures titles**

Figure 1. *Representation of the relative number of insects captured per flowers before (A) and during (B) invader flowering. Insect species are grouped by taxonomic order (and separating honeybees), and plants species by families (with an extra group for the invasive species). The size of the boxes represents the number of visits given or received by the group.*

Figure 2. *Model performance and variable contribution for the 18 reference models. Model performance is quantified by its proportion of variance explained:  $R^2$  (A & C), and variable relative contribution distinguishes between the ecological variables of interest (B & D, green gradient) vs. the variables linked to protocol choices (B & D, brown gradient).*

Figure 3. *Impact of the frequency of flower area measurement (once per period or once per sampling event) on the model performance ( $R^2$ ; A-C), and on the contribution of the ecological variables of interest (D-F), before and during invader flowering for all groups (A & D), per insect group (B & E), and per plant families (C & F).*

Figure 4. *Impact of ignoring observed but non-captured visits on model performance ( $R^2$ ; A-C), and on the accumulated contribution of the ecological variables of interest (D-F). The results are presented before and during invader flowering for all groups (A & D), per insect group (B & E), and per plant families (C & F).*