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EMMA JEAVONS (Orcid ID : 0000-0002-5934-7872)

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**Corresponding Author Email ID: emma.jeavons@ntymail.com**

## **Third and fourth trophic level composition shift in an aphid-parasitoid-hyperparasitoid food web limits aphid control in an intercropping system**

Emma Jeavons<sup>a, b, c</sup>, Joan van Baaren<sup>a</sup>, Anne Le Ralec<sup>d</sup>, Christelle Buchard<sup>e</sup>, Franck Duval<sup>e</sup>, Stéphanie Llopis<sup>a</sup>, Estelle Postic<sup>d</sup>, Cécile Le Lann<sup>a</sup>

<sup>a</sup> *University of Rennes, CNRS, ECOBIO [(Ecosystèmes, biodiversité, évolution)] - UMR 6553, Rennes, France*

<sup>b</sup> *Laboratoire de biologie végétale Yves Rocher, La Croix des Archers, 56200 La Gacilly, France*

<sup>c</sup> *LTSER « Zone Atelier Armorique », CNRS, Rennes, F-35043, France*

<sup>d</sup> *IGEPP, Institut Agro, INRAE, Université de Rennes 1, Université Bretagne-Loire, 35000 Rennes, France*

<sup>e</sup> *IGEPP, Institut Agro, INRAE, Université de Rennes 1, 35650 Le Rheu, France*

### **Abstract**

1. Understanding how resource diversification affects ecological interactions, food web structure and ecosystem functioning is essential in both fundamental and applied ecology. While plant diversification strategies (either in- or around-field) are often proposed in agricultural landscapes as practices to improve the biological control of herbivores by natural enemies, results remain variable and unsure.

2. Here, we studied the effect of an in-field diversification practice (the intercropping of leguminous crops within cereal fields, an increasingly common practice but with inconsistent results on biological control) on cereal aphid control and the structure of a cereal-aphid-parasitoid-hyperparasitoid food web for two years.

3. We report that aphid control was not increased in mixed fields, nor was cereal parasitoid diversity and food web complexity. Nevertheless, the provision of alternative hosts in mixed fields led to a functional community composition shift, favouring generalist parasitoid species over specialist ones.

4. Moreover, we observed a higher hyperparasitism rate in mixed fields, suggesting that secondary parasitoids were favoured by alternative resources, which may have disrupted aphid control by primary parasitoids.

5. *Synthesis and applications.* This study demonstrates that parasitoid community composition shift and increased top-down control by the fourth trophic level can impact parasitoid efficiency to control herbivores. These results highlight the necessity to study fine-scale mechanisms within food webs to be able to set-up efficient methods to support biodiversity and associated ecosystem services in agricultural landscapes.

**Key words:** alternative host; biological control; cereal crop; functional community composition; hyperparasitism; intercrop; leguminous crop

## Introduction

Habitat modification is undeniably one of the main anthropic factors impacting biodiversity and the functioning of ecosystems (Foley et al., 2005; Tylianakis et al., 2007). It not only affects the presence or absence of species, but also their interactions, impacting the structure of communities and food webs (Bartomeus et al., 2016; Rand et al., 2006; Tylianakis and Binzer, 2014). One reason is that modifying an environment alters the availability of resources, affecting trophic interactions (Albrecht et al., 2007; Tylianakis et al., 2007) as well as resource-related non-trophic interactions such as competition (Rand et al., 2007). Predicting the effect of diverse habitat alterations, both negative (e.g. habitat loss) and positive (e.g. diversification) on ecological interactions is therefore critical to sustain essential ecosystem functions and services.

The manipulation of agricultural habitats, such as in-field or around-field plant diversification, is often proposed as a solution to enhance biodiversity and its functions (Barnes et al., 2020; Benton et al., 2003). The Resource Specialization Hypothesis predicts that increased plant diversity will enhance higher trophic levels diversity by favouring species specialized on the additional resources (Hutchinson, 1959). Therefore, plant diversification is expected to support biological control of phytophagous pests in agricultural landscapes by the enhancement of their natural enemy populations due to a higher abundance and diversity of refugia, microhabitats and alternative food resources such as host/prey, pollen or nectar (the Enemy Hypothesis (Root, 1973; Shameer et al., 2018; Strong et al., 1984)). Plant diversification can also impact herbivore abundances through several bottom-up effects (Altieri and Letourneau, 1982; Poveda et al., 2008). For instance, the Resource Concentration Hypothesis (Root, 1973) predicts that host plants are more attractive, accessible and suitable for the development of specialist insect herbivores when plant diversity is

low. Diversified agricultural field should thus be less attractive to specialist crop pests while providing higher amounts of resources to their natural enemies and increase their control.

Nevertheless, despite those predictive theories, field studies show contrasted results, with either positive, neutral or negative effects of plant diversification on herbivore control (Heimpel, 2019; Letourneau et al., 2011; Poveda et al., 2008; Tscharrntke et al., 2016). Neutral or negative effects were suspected to be due to either a lack of natural enemy population increase (due for example to local disturbances or landscape context (Tscharrntke et al., 2016)) or to negative interactions among natural enemies (Straub et al., 2008) and higher trophic levels. Indeed, the dynamics of a population is predicted to depend on its trophic level position in the food web and the total number of trophic levels. In food webs comprising an even number of trophic levels, odd-numbered levels are expected to be limited by top-down forces (Fretwell and Barach, 1977; Shanafelt and Loreau, 2018). Nevertheless, higher trophic levels in plant-herbivore-natural enemy systems are still scarcely considered in biological programs (Rand et al., 2012). We argue here that considering the fine diet range of natural enemies and a more global food web approach, i.e. interactions within and between trophic levels, could boost our understanding of the mechanisms explaining the success or failure of different plant diversification strategies on biological control of pests. While most field studies on intra-guild interactions focus on predator systems, we chose to study a host-parasitoid system considering intra-guild interactions among primary parasitoid species (direct and indirect interspecific competition) and inter-guild interactions (hyperparasitism). In host-parasitoid systems, such questions are scarcely studied in natural conditions (Cusumano et al., 2016).

Here, we tested whether a resource diversification strategy, the intercropping of leguminous crops within cereal fields, impacted the control of cereal aphids by modifying the structure of an aphid-parasitoid-hyperparasitoid food web. Legume plants can provide carbohydrate food resources (Bugg et al., 1989) and alternative hosts (legume aphids) that could benefit primary parasitoids of the targeted herbivores (cereal aphids) but also secondary parasitoids (i.e. hyperparasitoids). Following the Enemy hypothesis, intercropping should lead to 1) a better top-down control, i.e. higher parasitism rates of cereal aphids and lower cereal aphid abundances in mixed crops compared to cereal crops. Several mechanisms at both the third and the fourth trophic levels could explain the reported aphid control. At the third trophic level, 2) intercropping should produce a more diverse parasitoid community with an enhancement of generalist parasitoid species using both cereal and legume aphids, which should lead to a complexification of the aphid-primary parasitoid trophic food web. On the other hand, at the fourth trophic level, 3) the addition of resources could foster secondary parasitoids and increase hyperparasitism rates, which could disrupt aphid control.

## **Material and methods**

### ***a) Study design***

The two-year study was conducted during spring 2018 and 2019, from mid-March to mid-June, corresponding to the main insect activity period. Cereal fields were either intercropped with leguminous crops (hereafter called “Mixed crops”; 23 fields: 10 in 2018 and 13 in 2019) or not (hereafter

called “Cereal crops”, 20 fields: 8 in 2018 and 12 in 2019). Cereals were commonly a mixture of triticale (*Triticosecale* spp.), oat (*Avena sativa* L.), spelt and/or wheat (*Triticum* spp). In intercropped fields, faba bean (*Vicia faba* L.) and pea (*Pisum sativum* L.) were mixed within cereals with no distinct row arrangement. Proportions of leguminous plants per field ranged from 16.7 to 55.6 % of the sowing density (See Table S1 in Supplementary Information). All fields were managed organically and were distributed across eastern Brittany (France) (Cartographic representation in Appendix S2; maximum distance between fields in 2018: 34 km; in 2019: 74 km).

### **b) Study organisms**

Three aphid species (Hemiptera: Aphididae) can be commonly found in cereal crops in western Europe: *Sitobion avenae* (Fabricius), *Metopolophium dirhodum* (Walker) and *Rhopalosiphum padi* (Linnaeus). These species are attacked by primary parasitoids from the Aphidiinae sub-family (Hymenoptera: Braconidae), mainly *Aphidius* species (*Aphidius avenae* (Haliday), *A. ervi* (Haliday), *A. matricariae* (Haliday), *A. rhopalosiphi* (De Stephani-Perez)), as well as *Ephedrus plagiator* (Nees) and *Praon volucre* (Haliday) (Powell, 1982). These parasitoid species vary in their host range: the oligophagous species *A. rhopalosiphi* is specialized on aphids developing on Poaceae (Powell, 1982) (in cereals: *S. avenae*, *M. dirhodum* and *R. padi*, hereafter referred as “the Poaceae specialist”). The others species are more polyphagous, as they develop on several aphid species attacking other plant families besides Poaceae (Raymond et al., 2016). In the rest of the manuscript, we will consider these species as “generalists”. Primary parasitoids are attacked by secondary parasitoids (hereafter referred as “hyperparasitoids”) mainly from the genera *Alloxysta*, *Phaenoglyphis* (Hymenoptera: Figitidae); *Dendrocercus* (Hymenoptera: Megaspilidae); *Asaphes*, *Pachyneuron* and *Coruna* (Hymenoptera: Pteromalidae) (Sullivan, 1987).

Faba beans and peas are attacked by other aphid species, among which *Acyrtosiphon pisum* (Harris) can be used as an alternative host by several generalist cereal aphid parasitoids: *A. ervi*, *A. avenae*, *E. plagiator* and *P. volucre* (Starý, 2009). *Aphis fabae* (Scopoli) can be found on faba bean plants and can be parasitized by *A. matricariae* (Barczak, 1990). Faba bean provides extra-floral nectar which is used as a carbohydrate resource by many parasitoid species (Bugg et al., 1989; Jamont et al., 2013).

### **c) Insect sampling**

During both years, aphid abundance on cereals was estimated during five sampling sessions from mid-March to mid-June (see Table 1 for sampling dates) by counting the number of live aphids on 50 randomly-selected cereal plants per field (mean of four shoots per plant, i.e. 200 shoots per field). Cereal aphid parasitism rate per field was estimated as the ratio between the number of mummified aphids and the total number of aphids per field. In 2018, this scoring was performed among the 50 plants sampled for cereal aphid abundance (see sample sizes in Table 1). In 2019, to increase the number of aphids sampled in a field, a specific scoring of 30 cereal plants bearing at least one aphid was performed eight times during the sampling season (Table 1). All encountered mummies were collected and placed in 1.5 mL Eppendorf tubes closed by a cotton ball and stored at room temperature until primary parasitoid or hyperparasitoid emergence. The emerged parasitoid and the aphid mummy were preserved in absolute alcohol for later identification.

To account for the variability in crop phenology between years, sampling sessions were pooled into three periods corresponding to the three main development stages of cereals: elongation (Period 1), heading (Period 2) and ripening (Period 3) (see specific sampling dates in Table 1).

To evaluate the use of alternative hosts by primary and secondary parasitoids, 267 mummified *A. pisum* were collected on pea and faba bean plants during both years and stored in similar conditions until parasitoid emergence (Table 1). As very few mummies of *Aphis* spp. (N = 26) were found in the field and none of them were parasitized by *A. matricariae*, we concluded that *A. pisum* was the major alternative host present in our system and *Aphis* aphids were discarded from the analyses.

#### **d) Insect identification**

Aphid mummies and parasitoids were identified morphologically under a binocular magnifying lens (Leica M125C, X100). Primary parasitoids were identified to the species level and hyperparasitoids were identified to the genus level (Powell, 1982). In 2018, due to unsuitable humidity conditions during storage, only 38% of the mummies emerged which led us to use a molecular approach for parasitoid identification of the un-emerged mummies. Sample DNA was extracted following a “salting-out” protocol (Sunnucks and Hales, 1996). We used a two-step diagnostic multiplex PCR using group and species-specific cytochrome oxidase I mtDNA primer pairs of species of interest in our system (protocol and primers available in Ye et al., 2017). In the first step, Aphidiinae and hyperparasitoid group-specific primer pairs were used which allowed to measure hyperparasitism rate. In the second step, primary parasitoid species-specific primer pairs were used to identify *A. avenae*, *A. ervi*, *P. volucre* and *E. plagiator*. *Aphidius rhopalosiphi* was not targeted in Ye et al. (2017), but as this species is dominant in our system, we also performed a singleplex PCR following Traugott et al. (2008) using the species-specific *A. rhopalosiphi* primer pair. *A. matricariae* is a paraphyletic species (Derocles et al., 2016) and cannot be detected with a single primer pair. As this species was poorly represented in the parasitoid community (mean of 1.7% during both years), it was discarded from the molecular analyses. There was a low detection of multiparasitism (several primary parasitoids species identified on a single sample) and the few samples (N=3) in which several species were detected were discarded. This molecular approach allowed to analyse 69.6% of the collected mummies for the first step (hyperparasitism rate), and 54.6% for the second step (primary parasitoid community).

Body size is often linked to higher foraging or competitive abilities and to a better fitness among parasitoids (Ellers et al., 1998). Despite differential competitive abilities inherent to species, developing in larger hosts may lead to a larger body size of parasitoids using both the focal and alternative hosts. Therefore, to understand if the higher proportion of generalists in mixed crops could be explained by their larger size, the sizes of the main generalist *A. ervi* and the Poaceae specialist *A. rhopalosiphi* from both crop types were compared. The methods and the results are presented in Appendix S3.

#### **e) Data analyses**

All statistical analyses were performed with R Studio (RStudio Team, 2018) v 3.6.1.

The effects of intercropping (Crop Type: Cereal crop and Mixed crop), development period (Periods 1, 2 and 3) and sampling year (2018 and 2019) were tested on cereal aphid parasitism rates and cereal aphid abundances with Generalized Linear Mixed Models (GLMMs). Models included the sampled field as a random factor to account for a correlation matrix between sampling sessions. Cereal aphid parasitism rates per field were analysed by creating a two-vector response variable binding the number of aphid mummies with the number of live aphids per field per period (*cbind* command) (Crawley, 2005) and with a binomial error distribution (link function: “logit”, function *glmer* from the “lme4” package (Bates et al., 2014)). Cereal aphid abundances per plant were analysed considering a quasi-Poisson error distribution (*glmmPQL* function from the “MASS” package, “log” link function) to deal with overdispersed count data.

Relative abundances of each aphid species, parasitoid species and hyperparasitoid genus were analysed by binding the abundance of the species/genus per field with the sum of the other species/genus abundances per field (Crawley, 2005). A similar method was used for the ratio between the generalist *A. ervi* and the Poaceae specialist *A. rhopalosiphi* (*A. ervi* abundance per field with *A. rhopalosiphi* abundance per field) as well as hyperparasitism rates (number of hyperparasitoids emerged per field with the number of primary parasitoids emerged per field). Fields with less than 5 samples were discarded from the analyses. Only dominant species (i.e. with a sufficient number of individuals) were analysed (*S. avenae* and *M. dirhodum* for the aphid trophic level; *A. ervi*, *A. avenae* and *A. rhopalosiphi* for the parasitoid trophic level; *Alloxysta* spp. and *Dendrocerus* spp. for the hyperparasitoid trophic level). For aphid species relative abundances, the effect of intercropping, development period and sampling year was analysed with a GLMM specified with a binomial error distribution, including the sampled field as a random effect. For parasitoid species relative abundances and hyperparasitism rates, GLMMs specified with a binomial error distribution were used to analyse the effect of intercropping and crop development period on the bound response variables in 2019, including the sampled field as a random effect. In 2018, for sample size purposes, the cumulative numbers over the whole sampling season were used and the effect of intercropping on the response variables was analysed with Generalized Linear Models (GLMs, *glm* function) specified with a quasibinomial distribution to account for overdispersed data. The effect of intercropping and crop development period on the relative abundances of each hyperparasitoid genus were analysed in 2019 with GLMs specified with a quasibinomial distribution. Comparisons of the proportions of each species/genus in each period were calculated with  $\chi^2$  tests (with a Yates' correction if needed).

Primary parasitoid Shannon diversity index per field and cereal aphid-parasitoid food web metrics (Connectance, Interaction evenness, Generality, Vulnerability) per field were generated respectively with the *diversity* function from the “vegan” package (Oksanen et al., 2019) and the *networklevel* function from the “bipartite” package (Dormann et al., 2008). For all calculations, fields with less than 5 samples were discarded from the analyses. In 2019, the effects of intercropping and crop development period were assessed on diversity and food web indices with Linear Mixed Models (LMMs, *lmer* function), including the sampled field as a random factor. In 2018, for sample size purposes, diversity and food web indices per field were calculated for cumulative individuals over the whole season, and the effect of intercropping was assessed with Student permutation tests (*perm.t.test* from the “RVAideMemoire” package, 999 permutations). Hyperparasitoid genus diversity and cereal aphid-hyperparasitoid food web complexity indices were calculated in 2019 with cumulative samples over the whole sampling season and the effect of intercropping was analysed with Student permutation tests. They could not be calculated in 2018 due to the small number of samples (Table 1).

For all models, the significance of each effect was tested with a Wald Chi-square test using a type II sum of squares (*Anova* function from the “car” package). Interactions between variables were tested in the models and removed if not significant. Post-hoc tests were used for pairwise comparisons with the *emmeans* function (“lme4” package (Bates et al., 2014)). Final models were checked to ensure they met model assumptions (Crawley, 2005).

## Results

### 1) Cereal aphid parasitism rate and cereal aphid abundances

Mean cereal aphid parasitism rate was of  $0.25 \pm 0.034$  (mean  $\pm$  standard error) in 2018 and  $0.10 \pm 0.0068$  in 2019 (Fig. 1.a). The effect of crop type on parasitism rate was moderated by the period and the year (i.e. the interaction between crop type, period and year had a significant effect on parasitism rate) (Table 2.a). Nevertheless, no difference in parasitism rates between crop types was detected for any period during both years (post-hoc results are given by the letters in Fig. 1.a). Parasitism rate increased throughout the season in 2018, and remained stable in 2019 (Table 2.a, Fig. 1.a).

Cereal aphid abundances had a mean of  $0.35 \pm 0.019$  aphids per plant in 2018 and  $0.51 \pm 0.038$  aphids per plant in 2019 (Fig. 1.b). The interaction between crop type, period and year had a significant effect on cereal aphid abundances (Table 2.a). No difference in aphid abundances was detected between crop types for neither period nor year (post-hoc results are given by the letters in Fig. 1.b). In 2018, aphid abundances remained similar among periods; in 2019, abundances increased at P3 (Table 2.a, Fig. 1.b).

### 2) Cereal aphid-primary parasitoid food web structure

The three main cereal aphid species (*S. avenae*, *M. dirhodum* and *R. padi*) were present in both cereal and mixed crops, and crop type did not influence their relative abundance (Table 2.a). *Sitobion avenae* was the dominant species during both years (2018, P1:  $\chi^2=295.5$ ;  $df=2$ ;  $p<0.001$ ; P2:  $\chi^2=105.2$ ;  $df=2$ ;  $p<0.001$ ; P3:  $\chi^2=244.7$ ;  $df=2$ ;  $p<0.001$ ; 2019, P1:  $\chi^2=158.5$ ;  $df=2$ ;  $p<0.001$ ; P2:  $\chi^2=332.0$ ;  $df=2$ ;  $p<0.001$ , P3:  $\chi^2=3219.9$ ;  $df=2$ ;  $p<0.001$ ), and its abundance increased along the season ( $64.0 \pm 5.5\%$  in P1,  $79.0 \pm 3.1\%$  in P2 and  $88.3 \pm 3.4\%$  in P3) (Table 2.a). *Metopolophium dirhodum* was the second most abundant species ( $24.9 \pm 4.5\%$  in P1,  $19.3 \pm 3.0\%$  in P2 and  $11.3 \pm 3.4\%$  in P3) (Table 2.a). *Rhopalosiphum padi* abundances decreased along the season and disappeared in P3 ( $11.1 \pm 2.8\%$  in P1,  $1.7 \pm 0.63\%$  in P2 and  $0.34 \pm 0.18\%$  in P3).

Cereal aphid parasitoid diversity was similar in both types of crops during both years (Table 2.b) with six species identified (Fig. 2) among which *A. rhopalosiphi*, *A. ervi* and *A. avenae* represented 90.6% of the community. In 2019, the Poaceae specialist *A. rhopalosiphi* was the dominant species during cereal elongation (P1,  $49.3 \pm 4.5\%$ ;  $\chi^2=172.9$ ;  $df=2$ ;  $p<0.001$ ) and decreased in proportion over time, disappearing at P3 ( $3.4 \pm 1.3\%$ ) (Table 2.b, Fig. 2, Fig. 3). *Aphidius ervi* became dominant at P2 in mixed crops ( $46.3 \pm 3.7\%$ ;  $\chi^2=19.6$ ;  $df=2$ ;  $p<0.001$ ) and *A. avenae* at P3 ( $56.9 \pm 5.5\%$ ;  $\chi^2=542.1$ ;  $df=2$ ;  $p<0.001$ ) (Table 2.b, Fig. 2, Fig. 3). A similar pattern was observed in 2018 (Fig. 2). The proportion of the Poaceae specialist *A. rhopalosiphi* was significantly lower in mixed crops in 2018 compared to cereal crops but not in 2019 (Table 2.b, Fig. 3 for 2019). During both years, the proportion of the generalist *A. ervi* was significantly higher in mixed crops compared to cereal crops whereas the proportion of *A. avenae*



was not affected by crop type (Table 2.b). The ratio between *A. ervi* and *A. rhopalosiphi* abundances per field was significantly higher in mixed crops compared to cereal crops during both years (Table 2.b). In 2019, the ratio increased over time, indicating a reduction of the proportion of *A. rhopalosiphi* compared to *A. ervi* along the season (Table 2.b, Fig. 3). Connectance, interaction evenness, generality and vulnerability indices were not affected by crop type for either year (Table 2.b, Table S4). There was no effect of crop type on *A. ervi* nor *A. rhopalosiphi* female sizes (see Appendix S3).

The generalist species *A. ervi* represented 71.7% of the primary parasitoids emerging from *A. pisum* (N=152 among the 212 emerged primary parasitoids). *Aphidius avenae* and *P. volucre* emerged from respectively 4.2% (N=9) and 2.4% (N=5) of the *A. pisum* mummies. Other parasitoids emerging from *A. pisum* (21.7%, N=56) were non-cereal aphid parasitoids such as *Aphidius eadyi* and *Aphidius banksae*.

### 3) Fourth trophic level and cereal aphid-hyperparasitoid trophic food web structure

In 2018, no differences in hyperparasitism rates between mixed and cereal crops were recorded, whereas in 2019, hyperparasitism rates were significantly higher in mixed crops and increased during the season (Table 2.b, Fig. 4). In 2019, four hyperparasitoid genera were recorded on both crop types on cereal aphids: *Alloxysta* spp., *Asaphes* spp., *Dendrocerus* spp. and *Phaenoglyphis* spp. (Fig. 5). Two individuals of *Coruna* spp. were also recorded on mixed crops only. *Alloxysta* spp. was the dominant genus during P1 ( $95.0 \pm 5.0\%$ ;  $\chi^2=14.5$ ;  $df=4$ ;  $p<0.001$ ) and P2 ( $80.1 \pm 6.3\%$ ;  $\chi^2=136.2$ ;  $df=4$ ;  $p<0.001$ ), while *Dendrocerus* spp. was the dominant genus at P3 ( $71.0 \pm 3.5\%$ ;  $\chi^2=467.4$ ;  $df=4$ ;  $p<0.001$ ) (Table 2.b, Fig.5). Neither genera diversity nor food web metrics were affected by crop type (Table 2.b). *Acyrtosiphon pisum* individuals were hyperparasitized by the same hyperparasitoid genera, but hyperparasitism rate was low ( $0.08 \pm 0.025$ ).

## Discussion

Contrary to our first hypothesis, intercropping did not increase biological control in our studied system. To explain this result, we propose to examine the effect of the additional resources on the third and the fourth trophic levels. Primary parasitoid diversity was not enhanced by the addition of resources, and did not lead to higher food web complexity contrary to our second hypothesis. Nevertheless, our results report a shift in the primary parasitoid composition, with the most generalist species being dominant in mixed crops. As predicted by our third hypothesis, hyperparasitism was increased in mixed crops, which could neutralize the benefits of increased resource availability by intercropping and explain the absence of increased aphid control.

### Effect of intercropping on cereal aphid parasitism rates and abundances

A wide variety of intercropping systems have been studied and/or implemented worldwide, and show generally positive results on natural enemy enhancement and resulting biological control. Intercropping systems involving wheat crops usually lead to a lower pressure of herbivores due to the lower density of host plants (Lopes et al., 2016). Our results differ from this general trend and Root's (1973) Resource Concentration Hypothesis, as the abundance of the herbivore in our system was similar in both crop types (for the same number of cereal plants) during both years, showing no global effect of additional resources on herbivore pressure. Moreover, intercropping systems with wheat scarcely enhanced natural enemies in the

literature (measured by the abundance, diversity or activity-density) (Lopes et al., 2016), which was the case in our study (here, parasitism rate). Nevertheless, our results showed that the different species of our system were impacted differently by increased resource availability. We propose several mechanisms which might explain why intercropping did not foster biological control of herbivores in this system, at the parasitoid trophic level and the hyperparasitoid trophic level.

Independently of crop type, in 2018, parasitism increased along the season, which might explain the steady abundance of aphids observed, suggesting an efficient control. Interestingly, the opposite pattern was observed in 2019, with a steady parasitism rate along the season and increased aphid abundances, showing a weaker control. These results highlight the importance of parasitism for herbivore control all along the season (Ortiz-Martínez et al., 2019).

### **Effect of intercropping on the third trophic level**

The addition of leguminous plants led to the colonization of other herbivore species specialized on those crops such as *A. pisum*, as predicted by the Resource Specialization Hypothesis (Hutchinson, 1959). This increased richness in aphids projected into an increased richness of parasitoids, with the attraction of species specialized on those aphid species (*A. eaydi*, *A. banksae*). Nevertheless, contrary to our second hypothesis, resource diversification did not increase cereal aphid parasitoid richness, probably because cereal crops already attracted all existent parasitoid species that could parasitize cereal aphids from the environment (Vollhardt et al., 2008). Diversity was also not enhanced, although there was a shift in the proportion of each species. Indeed, as predicted, resource addition promoted the relative abundance of the main generalist parasitoid (*A. ervi*) over the main specialist parasitoid (*A. rhopalosiphi*). This result is consistent with other studies that showed that generalist species are favoured when the targeted host/prey is scarce if they can develop on other resources in the environment (Rand and Tscharrntke, 2007). Nevertheless, most of these studies compare predators as the generalist natural enemies and parasitoids as the specialist natural enemies, whereas in our study we show that this pattern stays accurate at a much finer scale, within the parasitoid community.

Such a functional community shift may be driven by bottom-up effects impacting species differently. The specialist species (here, *A. rhopalosiphi*) may be disfavoured in more diverse fields due to a dilution of its specific resource (here, cereal aphids) (Lopes et al., 2015). Such a dilution effect may have impacted the specialist attraction and establishment in mixed fields (Root, 1973). On the contrary, the main generalist parasitoid may be enhanced due to more abundant and profitable resources in mixed crops. Here, the presence of *A. pisum* on leguminous plants and the ability of *A. ervi* to transfer from *A. pisum* to *S. avenae* (Cameron et al., 1984; Daza-Bustamante et al., 2002) could have participated in increasing the attractivity of mixed crops to *A. ervi* and its development. Moreover, *A. ervi* has co-evolved with the host-plant complex Fabacea – *A. pisum*, which is very attractive for this species (Luquet et al., 2019). Therefore, *A. ervi* may have a better capacity to detect and use Fabacea extra-floral nectar than a cereal habitat specialist.

The community shift may also be driven by competitive interactions among parasitoid species, impacting the lower competitor's fitness and population development (Cusumano et al., 2016). The generalist *A. ervi* is known to be a superior competitor compared to *A. rhopalosiphi* (Le Lann et al., 2008; Ortiz-Martínez et al., 2019) and to preferentially attack hosts parasitized by *A. rhopalosiphi* compared to sane hosts when the host

resource is scarce (*i.e.* multiparasitism) (Ortiz-Martínez et al., 2019). *Aphidius ervi* may thus have limited the development of *A. rhopalosiphi*, especially in mixed fields where the generalist was favoured. In addition, we checked if the presence of larger hosts (here, *A. pisum*) in mixed fields increased the generalist species size, that is directly related to its competitiveness (Ellers et al., 1998; Sagarra et al., 2001). Our results did not support this hypothesis, as intercropping did not impact *A. ervi* size. Interestingly, *A. ervi* individuals were always larger than *A. rhopalosiphi* individuals for mummies that were the same size, which could ensue from an energetic gain of multiparasitism. Indeed, *A. ervi* is known to gain weight from superparasitized hosts (Bai and Mackauer, 1992).

The functional community composition shift may have impacted the efficiency of the community to control cereal aphids, as *A. rhopalosiphi* is expected to be more efficient in finding and parasitizing cereal aphids compared to generalist species (Raymond et al., 2016). In a comparable study, the proximity of mustard crops, which provides nectar but no alternative hosts for *A. ervi*, increased parasitism rate of cereal aphids, and *A. rhopalosiphi* was the main parasitoid in this system (Damien et al., 2017). Therefore, the presence of alternative hosts may have hindered the effect of nectar resources on the specialist parasitoid populations and limited the efficiency of the community to control aphids in our legume-diversified system.

Another hypothesis found in the literature which could explain why herbivore control is not increased in intercropping systems is the failing of the alternative resources provided to increase natural enemy populations (Lopes et al., 2016). Nevertheless, in our system, alternative hosts were used by *A. ervi*. Moreover, a recent study suggested that this same species could use faba bean extra-floral nectar in the field (Luquet, 2018). Faba bean extra-floral nectar is known to increase the longevity and the length of the oviposition period in the laboratory of several hymenopteran parasitoid species (Baggen et al., 1999; Jamont et al., 2013). Therefore, both host and nectar resources could have been used by at least one species in this system, but did not lead to increased biological control.

Independently of the crop type, our study highlighted a drastic shift in the composition of the parasitoid community in a single growing season (3 months), with the specialist (*A. rhopalosiphi*) being the predominant species at the beginning of the season, then replaced by more generalist species (*A. ervi* and *A. avenae*). Food web complexity was also altered along the season. Studying food webs and community composition at high temporal resolution is therefore essential to understand and/or predict resultant biological control along the season (Lohaus et al., 2013; Vollhardt et al., 2008). In practice, such understanding may have implications for adjacent vegetation management, for instance to favour generalist or specialist natural enemy species at different period in the season in order to maximise community efficiency.

#### **Effect of intercropping on the fourth trophic level**

Finally, our findings provide suggestive evidence that higher resource diversity may favour secondary parasitoid and dampen the effect of primary parasitoids on herbivores (Gagic et al., 2011; Rand et al., 2006). In 2019, hyperparasitism rate of cereal aphids was higher in mixed crops compared to cereal crops during the whole season. Both carbohydrate resources (Araj et al., 2008, 2009) and alternative host resources (Gagic et al., 2011; Rand et al., 2012; Van Nouhuys and Hanski, 2000) have been suggested to increase hyperparasitoid populations. In our study, *A. pisum* individuals were hyperparasitized by the same hyperparasitoid genera than cereal aphids, suggesting that they may serve as alternative hosts.

Such hyperparasitoid enhancement has been suggested to disrupt aphid control, either due to primary parasitoid mortality or, as suggested by Höller et al. (1993), dispersal of female primary parasitoids from patches containing a high hyperparasitoid density (Schooler et al., 2011). Such as for primary parasitoids, we observed a drastic change in the hyperparasitoid community composition along the season: more specialist hyperparasitoids (*Alloxysta* and *Phaenoglyphis*) (Gutierrez and Van Den Bosch, 1970; Singh and Srivastava, 1990; Sullivan, 1987) were dominant at the beginning of the season and broad generalists (*Dendrocerus* spp. in particular) (Chow and Mackauer, 1999; Sullivan, 1987) took over at the end of the season. Studying primary parasitoid-hyperparasitoid food webs along the season, for instance with molecular methods such as in Ye et al. (2017), would help to understand the mechanisms by which hyperparasitoids are enhanced in more diverse crops and the top-down effect on the parasitoid community and its efficiency. While the fourth trophic level is still poorly taken into account in biological programs, our results support the evidence that these interactions are important drivers in the structure of the food web and its functioning (Tougeron and Tena, 2019).

## **Conclusion**

The variability in the results of plant diversification strategies inhibits their implementation by farmers. Understanding the mechanisms underlying such results is thus crucial to improve those strategies. We suggest that diversity and food web complexity are not sufficient to predict the effect of resource diversification on biological control, in particular when the system is poor in terms of richness, and that a functional approach of the community composition (i.e. considering the fine diet range of parasitoids) and higher trophic levels are essential to consider. In our study, the addition of resources induced a functional shift of the primary parasitoid community due to the presence of alternative hosts which could be only used by the most generalist parasitoid. Such functional shift modified the community efficiency to control aphids, possibly due to direct and indirect intra-guild competition for host resources. While current knowledge on interspecific competition among parasitoids mainly focuses on laboratory studies (Cusumano et al., 2016), our study highlights the need for understanding the impact of parasitoid competition on top down control in natural conditions. Our findings also strengthen the hypothesis supported by several studies that higher resource diversity may also favour hyperparasitoids and dampen the effect of primary parasitoids on herbivores. Other resource diversification strategies, such as flowering strips or adjacent flower covers, provisioning nectar but not alternative hosts may be more efficient in similar systems to avoid intra- and inter-guild negative interactions. Each diversification strategy involves a specific system, and this study highlights the necessity to study such fine-scale mechanisms to be able to set-up efficient methods to support biodiversity and associated ecosystem services in agricultural landscapes.

## **Authors' contributions**

CLL, JvB, ALR and EJ conceived the study. EJ, CLL, JvB and SL performed the insect sampling, and EJ, SL, CB, EP and FD conducted the identifications. EJ and CLL analyzed the data. EJ, CLL and JvB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

## **Conflict of Interest**

The authors declare they have no conflict of interest.

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## Data Availability Statement

Data available via the Dryad Digital Repository <https://doi.org/10.5061/dryad.d51c5b049> (Jeavons et al, 2021)

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## Tables and Figures

Table 1: Overview of the sampling methods used in 2018 and 2019 to collect the different variables, the number of sampling sessions pooled into the three periods (P1: cereal elongation; P2: cereal flowering; P3: cereal milk-ripening), the dates of the sampling sessions (number of the week from the 1<sup>st</sup> of January of the sampled year), and the total number of individuals collected and identified at each period in cereal and mixed crops.

Table 2a. Statistical results of the models used to analyse the effect of crop type, period and year on aphid parasitism rate, abundance and aphid community composition (GLMMs). Significant effects ( $p < 0.05$ ) are highlighted in grey.

Table 2b. Statistical results of the tests and models used to analyse the effect of crop type and period on cereal aphid-parasitoid food web complexity (Student permutation tests in 2018; LMMs in 2019), parasitoid community composition (GLMs in 2018; GLMMs in 2019), cereal aphid-hyperparasitoid food web complexity in 2019 (Student permutation tests), hyperparasitism rates (GLM in 2018; GLMM in 2019) and hyperparasitoid community composition in 2019 (GLMs). Significant effects ( $p < 0.05$ ) are highlighted in grey.

Figure 1. Distribution of a. mean parasitism rates per field and b. mean cereal aphid abundances per plant in cereal crops (light grey) and mixed crops (dark grey) during the three crop development periods (P1: cereal elongation; P2: cereal heading; P3: cereal milk-ripening) in 2018 (left) and 2019 (right). Horizontal lines represent the median and white squares represent the mean. Statistical results of the pairwise comparisons of the triple interaction Crop type: Year: Period are represented by the letters. Sample sizes are given in Table 1.

Figure 2. Cereal aphid-primary parasitoid trophic food webs from pooled data in cereal crops (left) and mixed crops (right), at the three sampling periods (Period 1: cereal elongation; Period 2: cereal flowering; Period 3: cereal milk-ripening) in 2018 (left) and 2019 (right). Lower bars represent relative abundances of the three aphid species, while upper bars represent relative abundances of each parasitoid species. The arrow width represents the frequency of the trophic interaction. N is the number of aphid-parasitoid couples used to build the trophic webs.

Figure 3. Mean proportions per field of *A. ervi*, *A. avenae* and *A. rhopalosiphi* in cereal crops (light grey) and mixed crops (dark grey) at the three sampling periods (Period 1: cereal elongation; Period 2: cereal flowering; Period 3: cereal milk-ripening) in 2019. The error bars represent the standard deviation of the mean. Statistical models were performed on each species separately (see Table 2.b).

Figure 4. Distribution of the mean hyperparasitism rate per field in cereal crops (light grey) and mixed crops (dark grey) in 2018 (left) and 2019 (right). In 2018, data was pooled over the crop development periods. In 2019, the distribution is shown depending on the three crop development periods (P1: cereal elongation; P2: cereal heading; P3: cereal milk-ripening). Horizontal lines represent the median and white squares represent the mean. Significant differences between crop types are represented with asterisks: \*: p-value < 0.05; \*\*: p-value < 0.01; \*\*\*: p-value < 0.001. Sample sizes are given in Table 1.

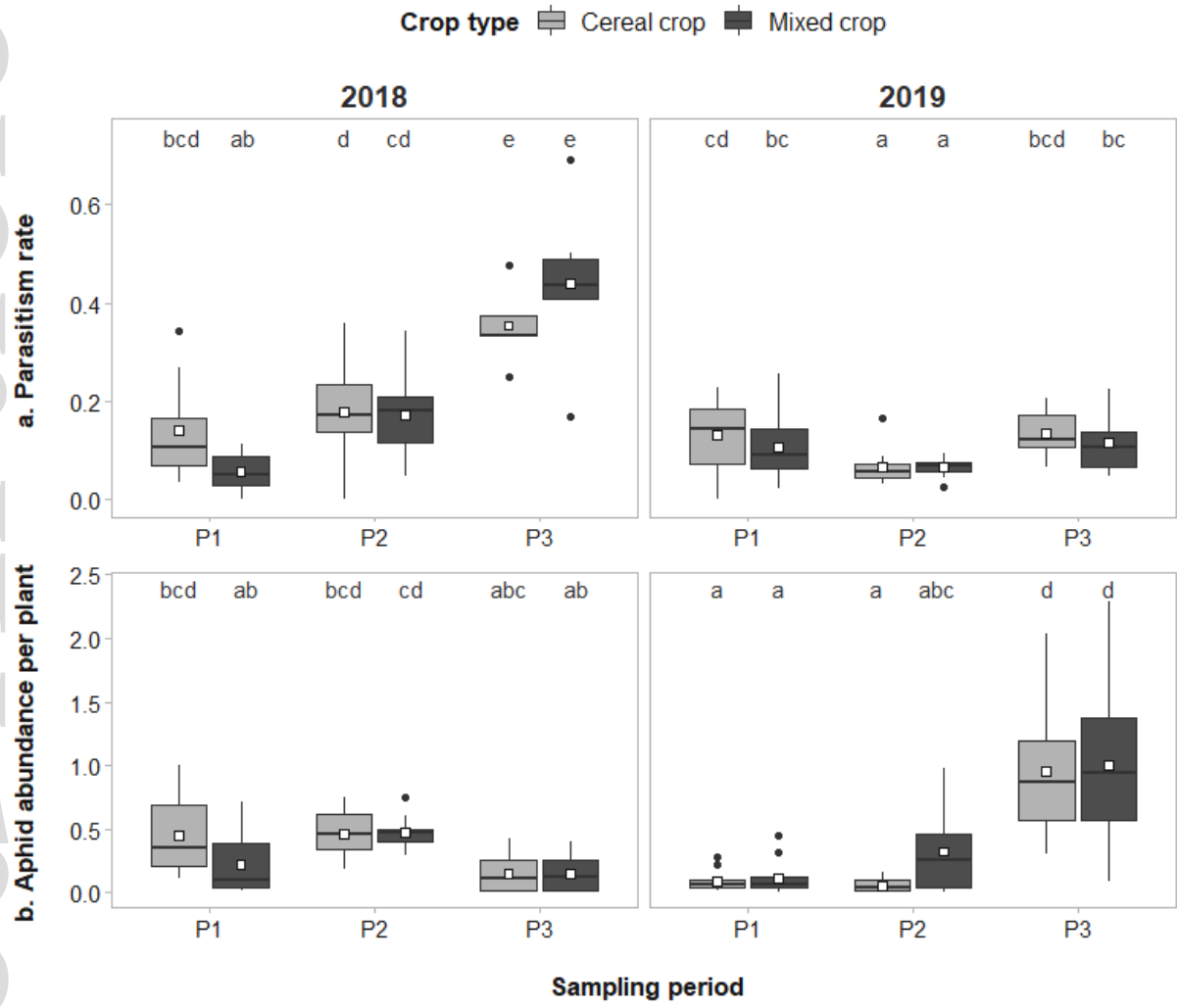
Figure 5. Cereal aphid-hyperparasitoid trophic food webs in cereal crops (left) and mixed crops (right), at the three sampling periods (Period 1: cereal elongation; Period 2: cereal flowering; Period 3: cereal milk-ripening) in 2019. Lower bars represent relative abundances of the three aphid species, while upper bars represent relative abundances of each hyperparasitoid genus. The arrow width represents the frequency of the trophic interaction. N is the number of aphid-hyperparasitoid couples used to build the trophic webs.

	2018					2019					
Variable	Method	Period	Sampling sessions	N		Method	Period	Sampling sessions	N		
				Cereal crop	Mixed crop				Cereal crop	Mixed crop	
Cereal aphid abundance	Prospection on 50 plants of cereal	P1	Week 12	122	32	Prospection on 50 plants of cereal	P1	Week 12	14	68	
			Week 15	239	187			Week 15	63	56	
			Week 18	250	314			P2	Week 20	30	209
			Week 21	119	158					Week 25	592
P3	Week 24	59	75	P3	Week 23	460	805				
Cereal aphid parasitism rate	Prospection on 50 cereal plants, calculation on plants bearing at least 1 aphid	P1	Week 12	122+7	32+2	Prospection on 30 plants bearing at least 1 aphid	P1	Week 12	136+23	144+12	
			Week 15	239+44	187+10			Week 15	411+82	479+83	
			Week 18	250+79	314+66			P2	Week 20	608+42	1092+67
			Week 21	119+17	158+28					Week 21	940+56
P3	Week 24	59+40	75+67	Week 22	1297+82	1391+89					
Aphid-parasitoid-hyperparasitoid trophic foodwebs	Collection of mummies encountered on the 50 prospected plants Morphological and molecular analysis identification	P1	Week 12	NA	NA	Collection of at least 10 mummies per field (or stop after 1h30) Morphological identification	P1	Week 12	34/30/0	25/19/0	
			Week 15	34/34/2	16/16/0			Week 15	132/77/2	120/83/4	
			Week 18	71/33/6	67/48/0			P2	Week 20	101/70/12	137/89/18
			Week 21	15/6/1	23/14/3					Week 21	124/88/5
P3	Week 24	17/6/18	29/14/29	Week 22	120/81/5	175/122/17					
Use of alternative hosts	Collection of pea aphid mummies on pea and faba bean plants	Number of collected mummies/identified primary parasitoids/identified hyperparasitoids			Number of collected mummies/identified primary parasitoids/identified hyperparasitoids						
		28/18/1			239/194/16						

a.	Crop type			Year			Period			Crop type:Year			Crop type:Period			Year:Period			Crop type:Year:Period			Field
	$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	SD (standard deviation)
<b>Parasitism rate</b>	2.51	1	0.113	84.5	1	<0.001	167.01	2	<0.001	0.39	1	0.531	7.88	2	0.019	99.33	2	<0.001	6.53	2	0.038	0.288
<b>Aphid abundance</b>	0.02	1	0.894	0.55	1	0.458	95.38	2	<0.001	5.29	1	0.021	12.56	2	0.002	106.72	2	<0.001	6.24	2	0.044	0.382
<b>Proportion of <i>S. avenae</i></b>	1.69	1	0.193	0.24	1	0.624	16.11	2	<0.001							9.42	2	0.009				0.856
<b>Proportion of <i>M. dirhodum</i></b>	2.17	1	0.141	0.99	1	0.320	6.28	2	0.043							9.38	2	0.009				0.840

b.

		2018			2019							
Third trophic level		Crop type			Crop type			Period			Field	
		$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	SD	
		Proportion of <i>A. ervi</i>	6.88	1	0.008	4.30	1	0.038	53.76	2	<0.001	0.385
		Proportion of <i>A. rhopalosiphi</i>	17.93	1	<0.001	2.35	1	0.125	243.83	2	<0.001	0.716
		Proportion of <i>A. avenae</i>	0.98	1	0.321	2.41	1	0.121	288.82	2	<0.001	0.877
		Ratio <i>A. ervi</i> / <i>A. rhopalosiphi</i>	6.26	1	0.012	5.13	1	0.024	110.76	2	<0.001	0.606
			t	p	$\chi^2$	df	p	$\chi^2$	df	p	SD	
		Parasitoid diversity	1.56	0.626	2.39	1	0.12	9.61	2	0.008	0.077	
		Connectance	-0.15	0.914	2.13	1	0.144	14.00	2	<0.001	0.018	
		Interaction evenness	0.004	0.974	0.30	1	0.583	51.91	2	<0.001	<0.001	
	Generality	-0.41	0.632	0.95	1	0.329	60.82	2	<0.001	0.178		
	Vulnerability	0.41	0.708	0.35	1	0.552	28.44	2	<0.001	0.152		
Fourth trophic level		$\chi^2$	df	p	$\chi^2$	df	p	$\chi^2$	df	p	SD	
		Hyperparasitism rate	0.04	1	0.837	4.03	1	0.045	69.52	2	<0.001	0.483
		Proportion of <i>Alloxysta</i> spp.				0.06	1	0.813	8.31	1	0.009	
		Proportion of <i>Dendrocercus</i> spp.				0.58	1	0.456	17.03	1	<0.001	
			t	p								
		Hyperparasitoid genera diversity				-0.59	0.556					
		Connectance				-0.54	0.548					
	Interaction evenness				-1.69	0.144						
	Generality				-0.74	0.424						
	Vulnerability				-0.29	0.724						





2018

Cereal crop

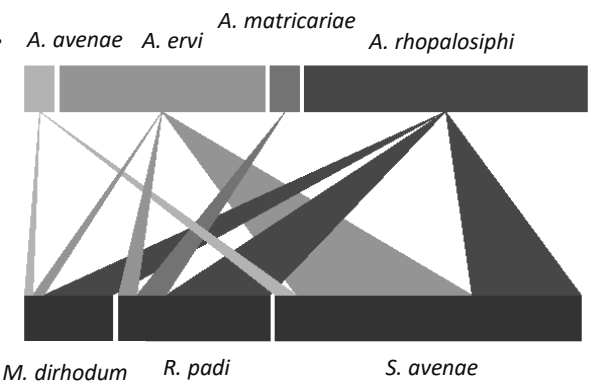
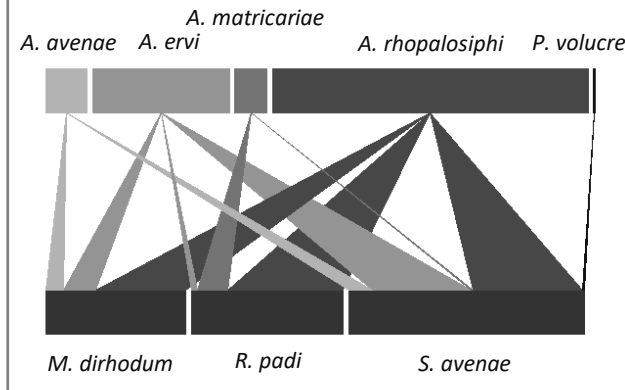
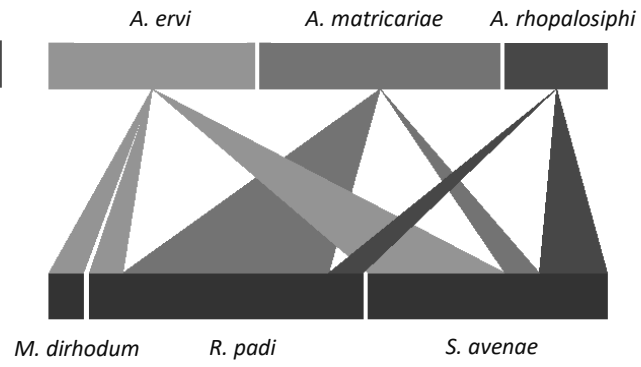
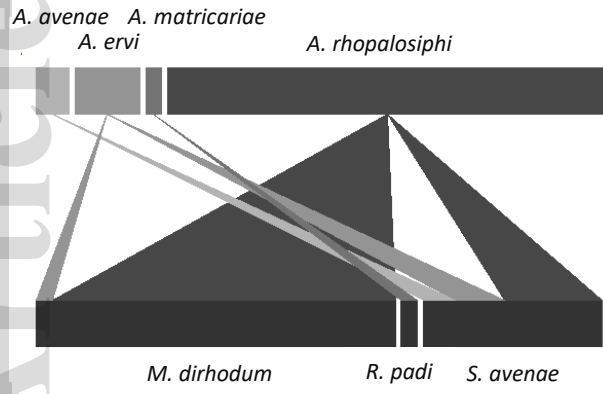
Mixed crop

2019

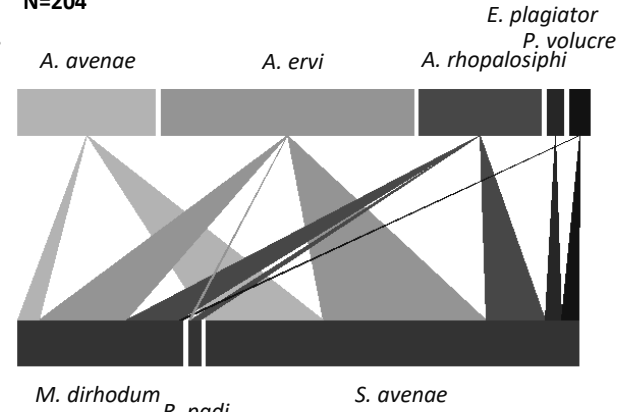
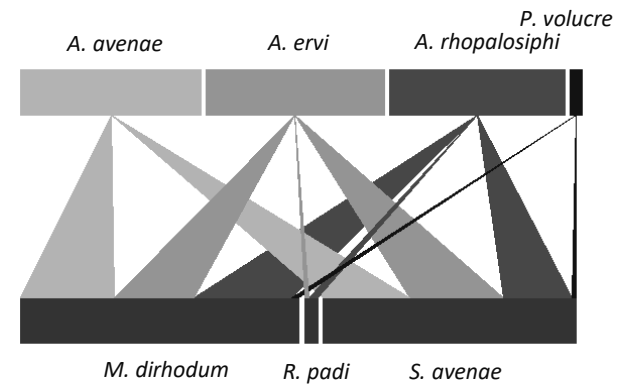
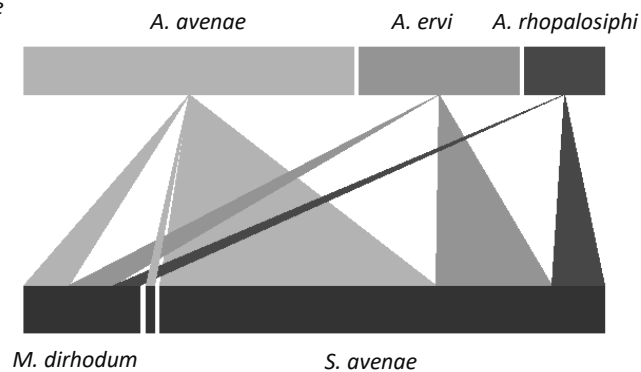
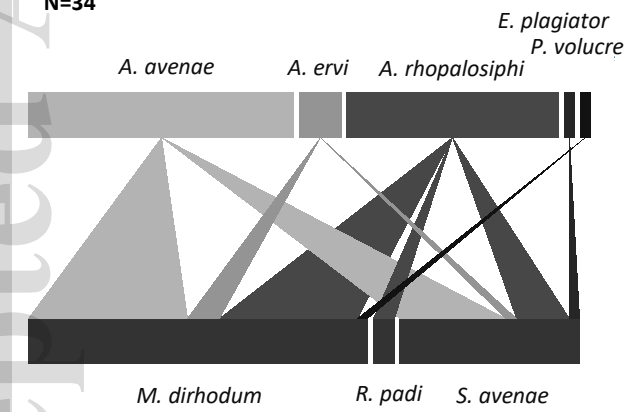
Cereal crop

Mixed crop

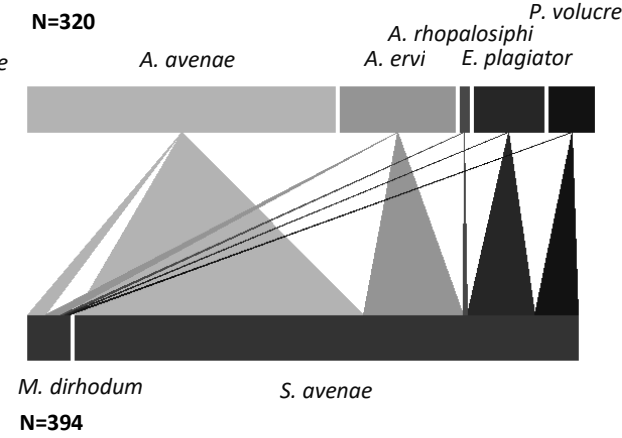
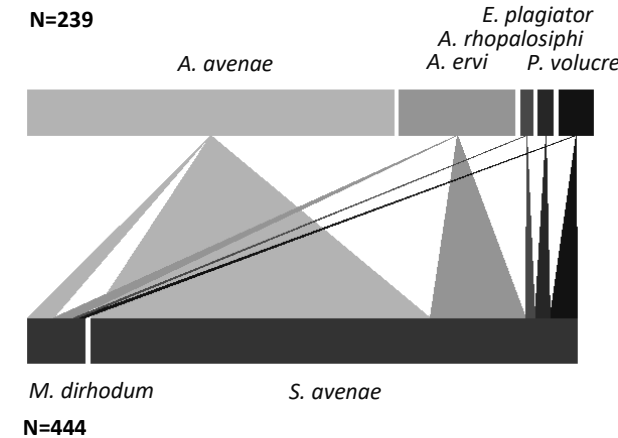
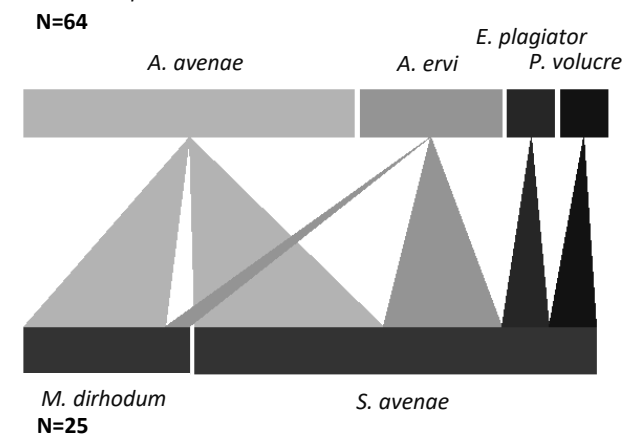
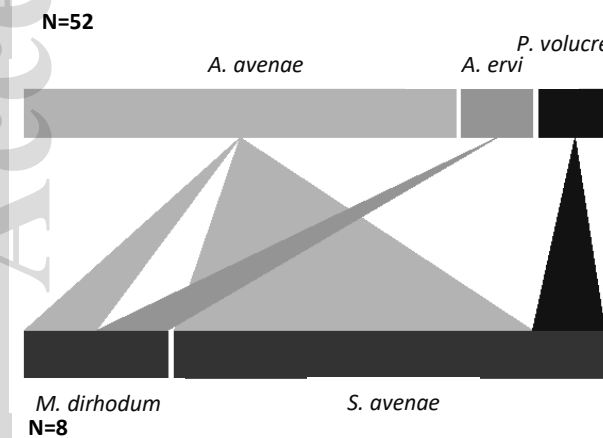
Period 1

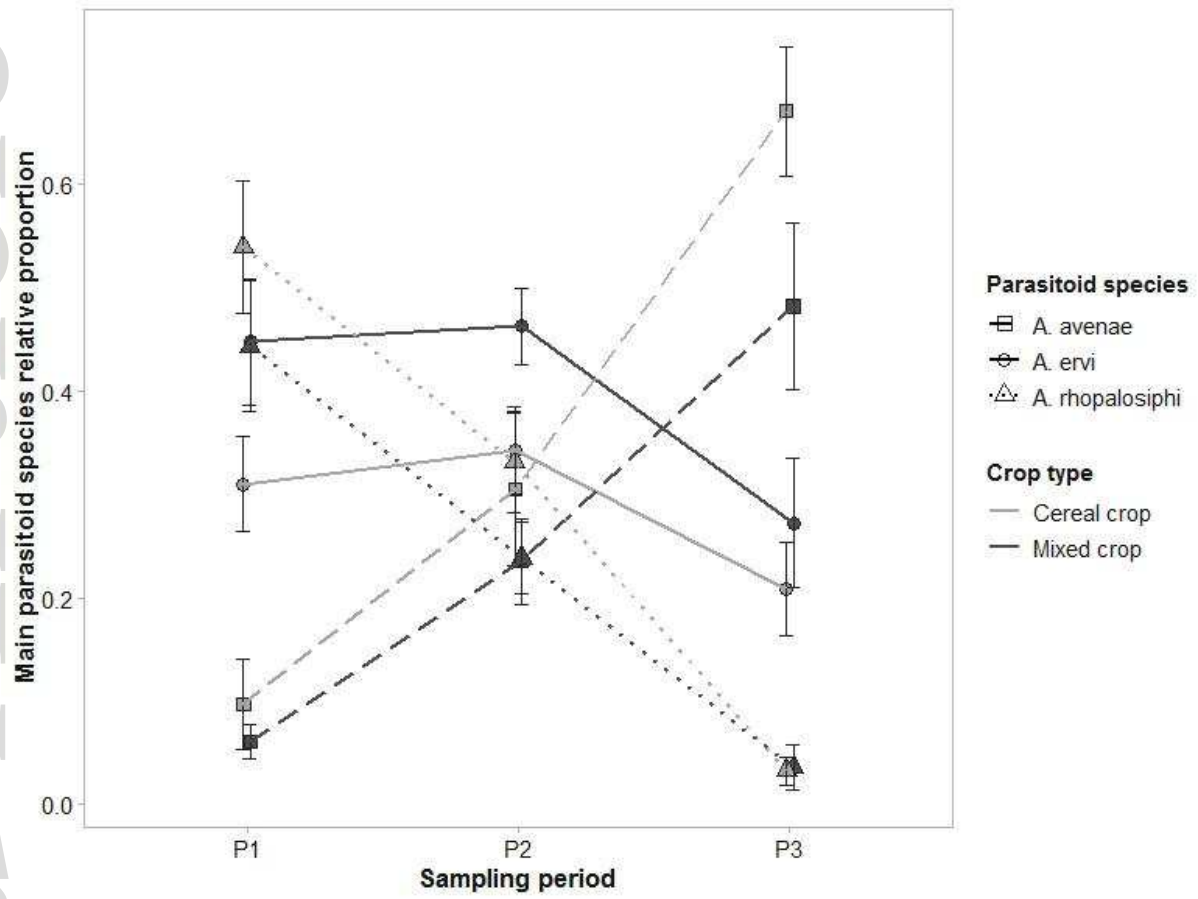


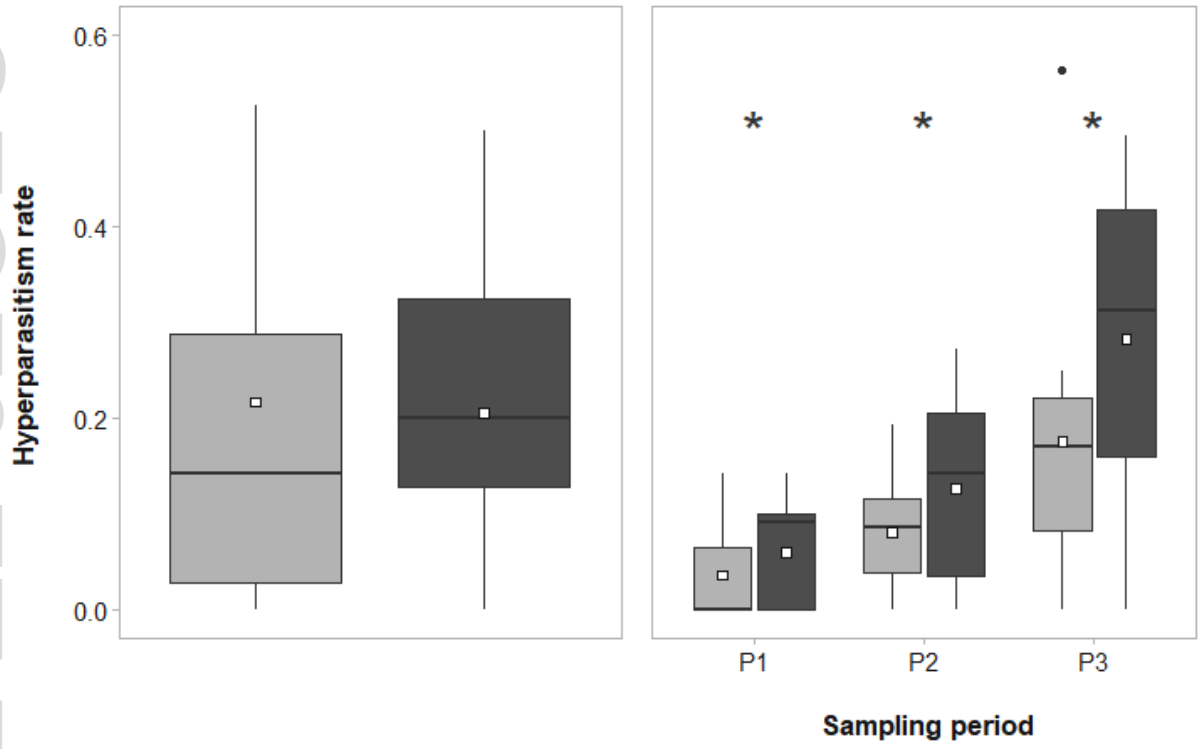
Period 2



Period 3



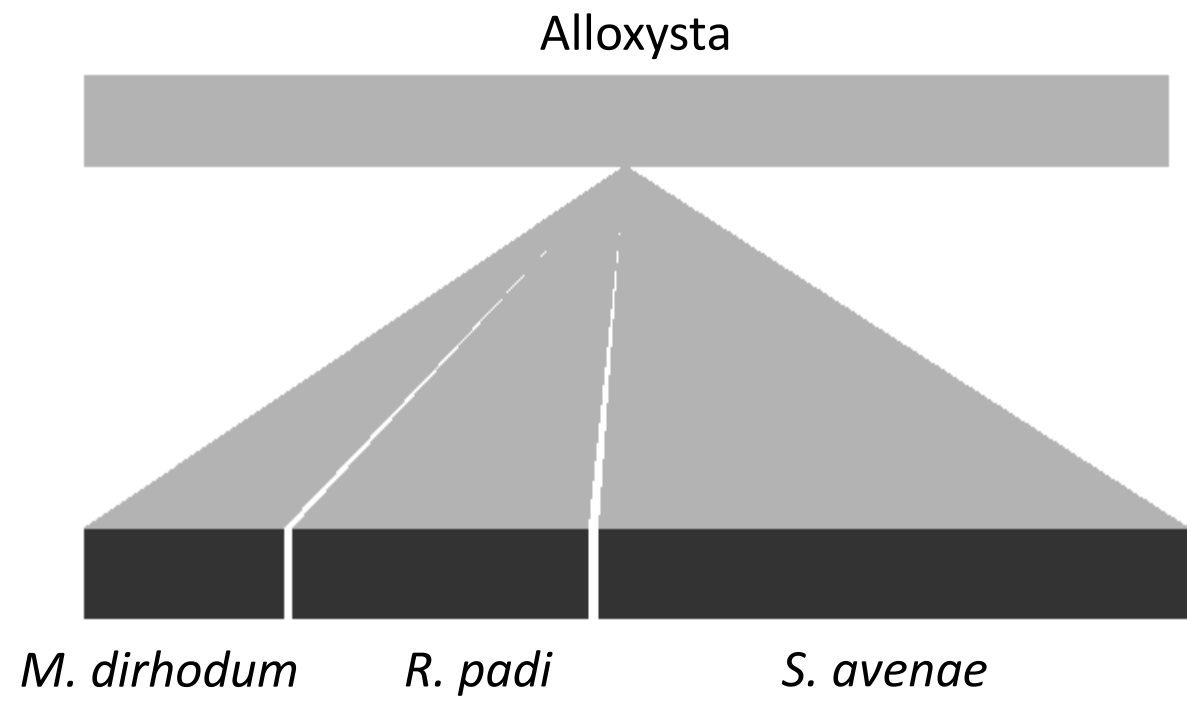
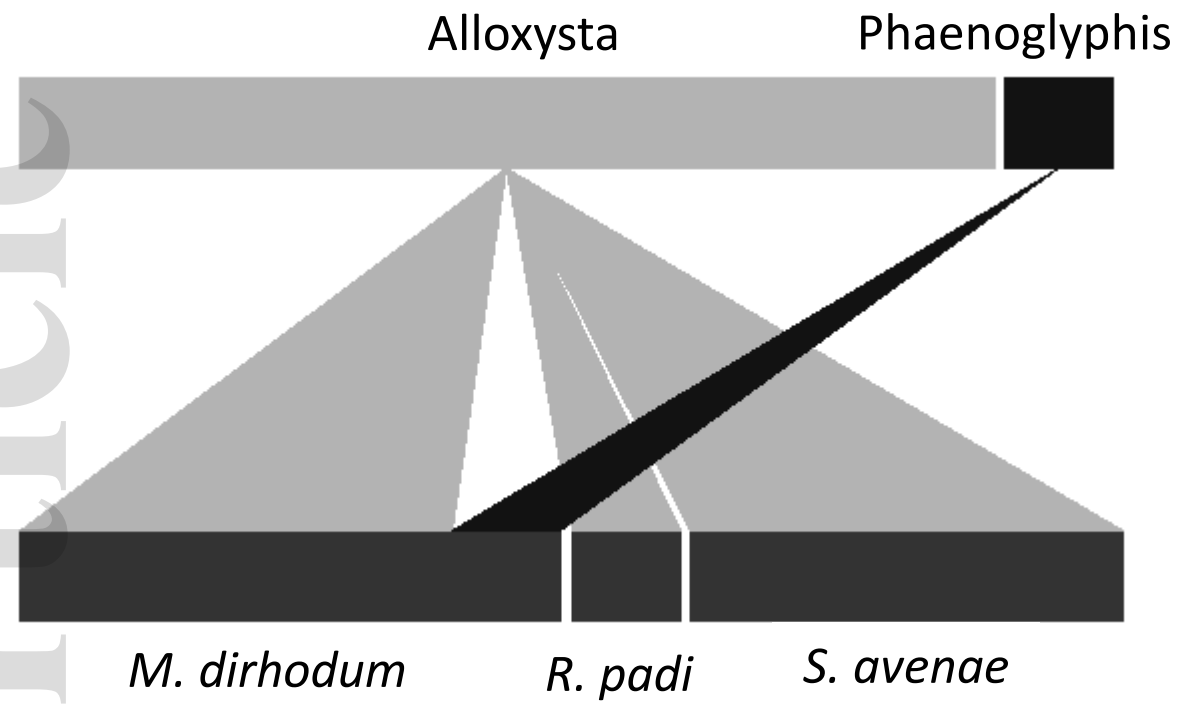




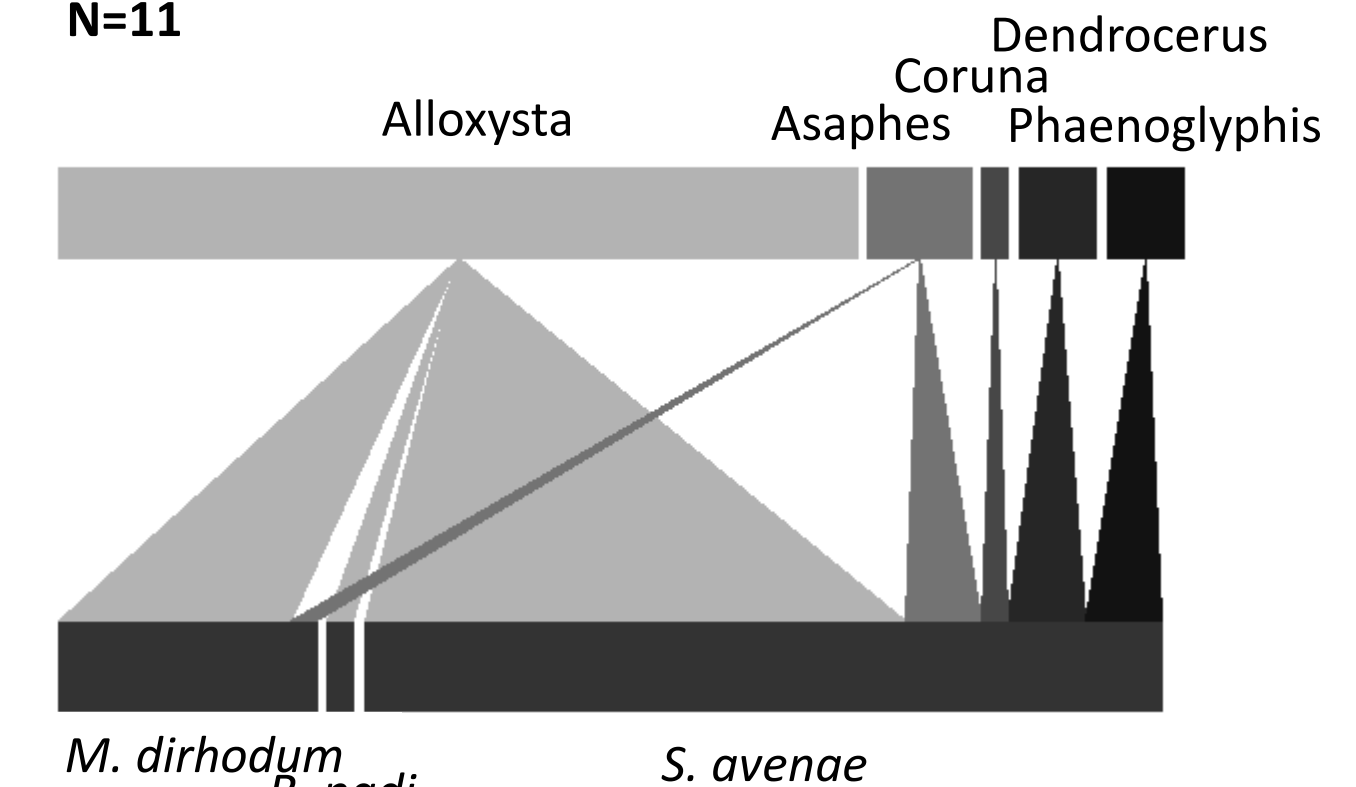
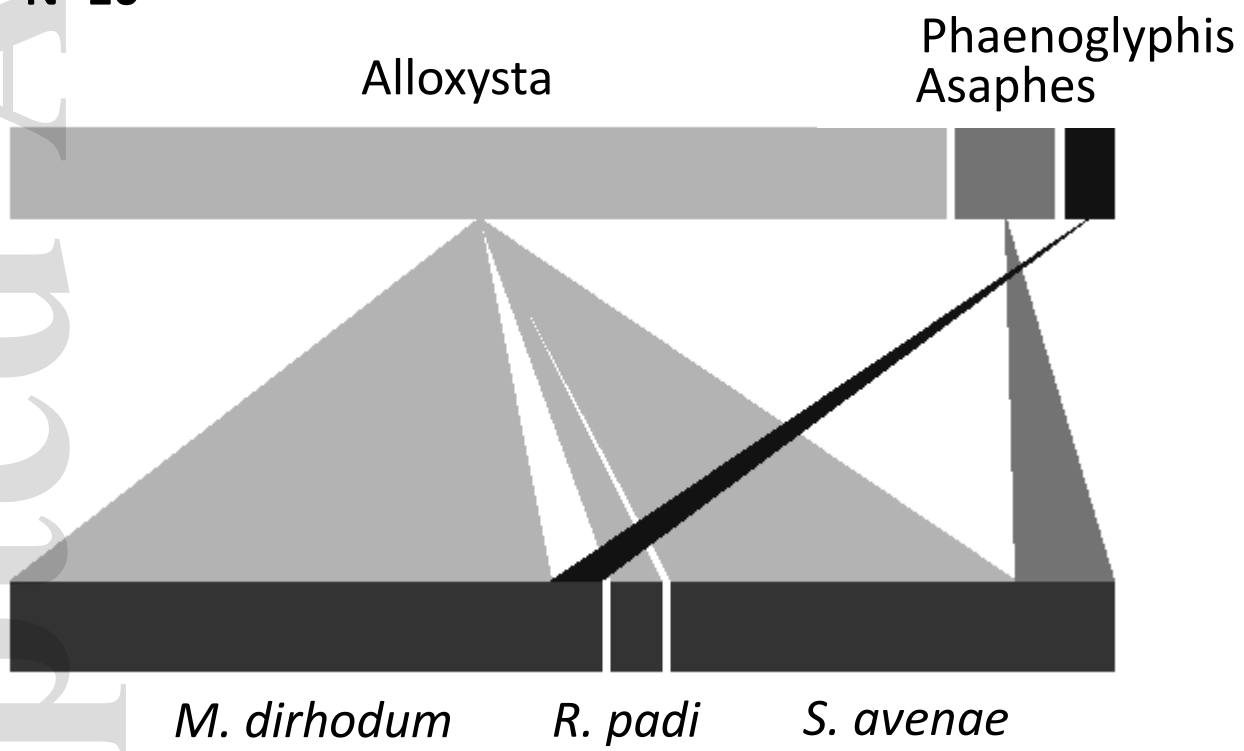
## Cereal crop

## Mixed crop

Period 1



Period 2



Period 3

