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Spatial and temporal heterogeneity in pollinator communities maintains within-species floral odour variation

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ABSTRACT

Flowering plants emit complex bouquets of volatile organic compounds (VOCs) to mediate interactions with their pollinators. These bouquets are undoubtedly influenced by pollinator-mediated selection, particularly in deceptively-pollinated species that rely on chemical mimicry. However, many uncertainties remain regarding how spatially and temporally heterogeneous pollinators affect the diversity and distribution of floral odour variation. Here, we characterized and compared the floral odours of ten populations of deceptively-pollinated *Arum maculatum* (Araceae), and inter-annual and decadal variation in pollinator attraction

within these populations. Additionally, we transplanted individuals from all sampled populations to two common garden sites dominated by different pollinator species (*Psychoda phalaenoides* or *Psycha grisescens*), and compared pollinator attraction rates to investigate whether populations maintained odour blends adapted to a specific pollinator. We identified high within- and among-population variation in a common blend of VOCs found across the range of *A. maculatum*. We also observed shifts in pollinator community composition within several populations over 1-2 years, as well as over the past decade. Common garden experiments further revealed that transplanted inflorescences generally attracted the dominant local pollinator species in both transplant sites. However, one population (Forêt du Gâvre, France) appears to exclusively attract *P. grisescens*, even when transplanted to a *P. phalaenoides*-dominated site. Together, our results suggest that maintaining diverse floral odour bouquets within populations may be advantageous when pollinator communities vary over short timescales. We propose that temporally-replicated ecological data are one potential key to understanding variation in complex traits such as floral odour, and in some cases may reveal resiliency to shifting pollinator communities.

KEYWORDS balancing selection, evolutionary ecology, floral volatiles, plant-pollinator interactions, reciprocal transplant, temporal variation

1. INTRODUCTION

Widely distributed species are subject to an array of eco-evolutionary settings across their range, due to variation in abiotic conditions and biotic interactions through space and time. These mosaics of selection are known to influence the traits that underpin diverse plant-herbivore and plant-pollinator interactions, affecting the evolutionary trajectories of all species involved (Levin 2000; Hendry 2017). Plant-pollinator interactions appear to be a particularly

important mechanism underlying the comparatively rapid rate of speciation in flowering plants (van der Niet & Johnson 2012; Hernández-Hernández & Wiens 2020); this macroevolutionary pattern is the result of microevolutionary processes acting on floral traits at the intraspecific level (Herrera et al. 2006). Understanding the evolutionary and functional ecology of complex floral traits therefore requires data on how plant-pollinator interactions vary through both space and time (Waser & Ollerton 2006). However, characterizing complex trait variation and biotic interactions across wide geographic ranges (e.g. Friberg et al. 2019) and through time (e.g. Fishbein & Venable 1996) requires substantial effort. As a result, few studies have simultaneously investigated the impact of spatial and temporal variation in pollinators on intraspecific floral trait variation.

As the dominant pollinators across all terrestrial ecosystems, insects exert key selective pressures on many floral traits. They may be attracted or repelled by odour (Whitehead & Peakall 2009), colour (Goyret et al. 2007; du Plessis et al. 2018), morphology (Ibanez et al. 2010, Murúa & Espíndola 2015), nectar (Parachnowitsch et al. 2019), pollen (Dobson & Bergström 2000), or the multimodal expression of several of these traits (reviewed in Junker & Parachnowitsch 2015). Here, we focus on floral odour bouquets, which are often complex blends of many volatile organic compounds (VOCs) that mediate diverse interactions between plants, insects, and microbes (Holopainen 2004; Raguso 2008).

Recent studies on plant-pollinator interactions have identified many cases of pollinator-driven evolution of floral odour (Chess et al. 2008; Klahre et al. 2011; Breitkopf et al. 2013; Peter & Johnson 2014; Gross et al. 2016). However, a review by Delle-Vedove et al. (2017) found that floral odour variation often cannot be explained by pollinator-mediated selection alone. Several factors may explain this discrepancy, including trade-offs between pollinator attraction and

chemical defence against herbivory (Schiestl et al. 2014), the nature of the interaction (*i.e.* deceptive pollination; Renner 2006), biochemical and energetic limitations (Delle-Vedove et al. 2011), phylogenetic constraints in the biosynthetic pathways for VOC production (Raguso et al. 2006), gene flow (Svensson et al. 2005), or genetic drift (Suinyuy et al. 2012). Another factor that may maintain floral odour variation within populations is temporal heterogeneity in pollinator community composition, as a result of pollinator declines (Yuan et al. 2009; Thomann et al. 2013; IPBES 2016; Zattara & Aizen 2021) or shifts in pollinator phenology (Burkle & Runyon 2019). However, the impact of temporally heterogeneous pollinators on floral odour is not yet clearly understood, because most of the aforementioned studies relied on VOC and pollinator data collected at a single timepoint. Here, we aim to address this gap in our knowledge, by examining how spatio-temporal variation in pollination interactions influences floral odour variation in *Arum maculatum* L. (Araceae).

Arum maculatum inflorescences (Figure 1) are pollinated deceptively, by emitting complex VOC blends which are known to vary within and among populations in England (Kite 1995; Kite et al. 1998; Diaz & Kite 2002), France (Chartier et al. 2011; Chartier et al. 2013), and across the Alps (Gfrerer et al. 2021). The floral odour of *A. maculatum* is similar to dung or decomposing organic matter (Lack & Diaz 1991), mimicking the natural brood sites of their main pollinators, the moth flies *Psychoda phalaenoides* L. and *Psycha grisescens* Tonnoir (Diptera: Psychodidae). Since pollinators are temporarily trapped in a specialized floral chamber (Bröderbauer et al. 2013) until the day after anthesis (Gibernau et al. 2004), it is possible to collect accurate quantitative data on pollinators attracted by individual inflorescences. Furthermore, pollinators appear to be attracted by VOCs alone (Dormer 1960; Urru et al. 2011), meaning that an inflorescence's reproductive success should be closely tied to its unique floral odour. Since *A. maculatum* floral odour is known to vary, and VOCs such

as indole, 2-heptanone, and p-cresol are attractive to *Psychoda* species (Kite et al. 1998), we chose to focus on floral odour in this study. Specifically, we investigated whether VOC variation is maintained within populations due to temporally variable pollinator communities, or across the species distribution due to spatial divergence in pollinator communities.

Floral odour variation may be maintained within populations through balancing selection (Delph & Kelly 2013) and/or phenotypic plasticity (Callaway et al. 2003; Majetic et al. 2009; Campbell et al. 2019). In the case of *A. maculatum*, balancing selection may occur if relative abundances of *P. phalaenoides* and *P. grisescens* vary temporally within populations (e.g. Schemske & Horvitz 1989), or if pollinator learning or avoidance to escape deception (Ayasse et al. 2000; Baguette et al. 2020) leads to frequency-dependent selection. In either case, we would expect to observe high floral odour variation within populations of *A. maculatum* across Europe. Phenotypic plasticity under variable abiotic and biotic conditions may also promote the maintenance of trait variation (Gulisija et al. 2016). If we observe shifts in VOC emissions when *A. maculatum* inflorescences are transplanted to non-native conditions, we may conclude that plasticity also contributes to the maintenance of floral odour variation.

Alternatively, if pollinators only vary spatially, they may exert divergent selective pressures on floral VOCs, leading to locally-adapted bouquets in populations with different pollinators (Leimu & Fischer 2008, Gervasi & Schiestl 2017; Suinyuy et al. 2018; Sayers et al. 2020). Previous research has shown that *P. phalaenoides* and *P. grisescens* are respectively trapped by inflorescences in the northern/central and southern/western portions of *A. maculatum*'s distribution (Espíndola et al. 2010) — except in the Atlantic fringe of France, where inflorescences trap *P. grisescens*. Furthermore, the population genetic structure of *A. maculatum* roughly aligns with this pattern (Espíndola & Alvarez 2011). Under spatially varying selection, we may predict that distinct *A. maculatum* floral VOCs or phenological

adaptations will be maintained in these two pollinator backgrounds. If these variations are local adaptations, we would expect to observe decreased pollinator attraction efficiency when inflorescences are transplanted to non-native pollinator backgrounds.

In this study, we investigated whether variation in *A. maculatum* floral odour was consistent with the patterns predicted under either of the two scenarios described above. To this aim, we surveyed variation in floral odour and pollinator attraction across Europe, and transplanted individuals from all sampled populations to two common garden sites dominated by either *P. phalaenoides* or *P. grisescens*. By selecting populations where pollinator communities were first surveyed a decade ago (Espíndola et al. 2010), we were further able to assess temporal heterogeneity in pollinators at both the inter-annual (*i.e.* our surveys between 2017-2019), and decadal scales, with the aim of understanding how temporally heterogeneous pollinators affect intraspecific floral odour variation. Our specific objectives were to (1) characterize and compare floral odour variation within and among populations of *A. maculatum*, (2) determine whether the pollinators trapped by *A. maculatum* vary spatially and/or temporally, and (3) use these results to infer potential drivers of floral VOC variation, namely pollinator-driven balancing selection and local adaptation.

2. MATERIALS AND METHODS

2.1 – Natural history & pollination ecology of Arum maculatum

Arum maculatum is a common European flowering plant, which is usually found in shaded areas of deciduous woodlands, particularly beech woods (Brown 2000). Its pollination cycle takes place over two days (reviewed in Gibernau et al. 2004). On the evening of the first day, the appendix is thermogenic and emits a dung-like odour to attract dipteran pollinators; heat alone is not sufficient to attract pollinators (Prime 1960), but likely aids in the dispersal of

VOCs such as indole, p-cresol, and 2-heptanone, which are attractive to *Psychoda* species when used in scented traps (Kite et al. 1998). Notably, the widely distributed *Psychoda phalaenoides* and *Psycha grisescens* are both trapped by *A. maculatum* across most of Europe, together representing 87.49% of all insects found within floral chambers (Espíndola et al. 2010). *Psychoda setigera*, *P. albipennis*, and *P. trinodulosa* are also infrequently observed within inflorescences, together representing 0.46% of trapped insects in Espíndola et al. (2010). Other infrequently attracted dipterans include Chironomidae (*Smittia pratorum*), Ceratopogonidae, and Sphaeroceridae (Brachycera) (Rohacek et al. 1990; Diaz & Kite 2002; Espindola et al. 2010).

Psychodidae are also known to be efficient carriers of *Arum* pollen (Albre et al. 2003; Diaz & Kite 2002); one individual can carry 50-150 grains of pollen, and a significant fruit set can develop from a single trapped pollinator (Lack & Diaz 1991). Based on their similar behaviours and morphologies, the above-mentioned *Psychoda* species are all presumably efficient pollinators of *A. maculatum*. However, antennal sensilla are known to vary among Psychodidae (Faucheux & Gibernau 2011), and species may therefore vary in their responses to VOCs. Although *A. maculatum* do not provide any nutritive rewards, this should not be detrimental to trapped *P. phalaenoides* and *P. grisescens*, as they do not feed during their short adult lifespan (P. Withers, cited in Lack & Diaz 1991). The conservation status of *P. phalaenoides* and *P. grisescens* has not been assessed; however, *Psychoda* populations are known to be large and dense (up to thousands of individuals per m^2; Arshad & Moh Leng 1991) due to their reliance on modern agriculture and human activities (e.g. manure and decomposing organic matter) for reproduction (Satchell 1947; Vaillant 1971). Peak *P. phalaenoides* abundances also appear to correspond with the peak flowering period of *A. maculatum* (Ollerton & Diaz 1999).

2.2 – Field sampling sites

We sampled ten populations of *A. maculatum* (Figure 1 & Appendix S1, Table S1), including three in France (Forêt du Gâvre, Conteville, and Chaumont), two in Switzerland (Neuchâtel and Cortaillod), one in Italy (Montese), one in Croatia (Visuć), two in Serbia (Gostilje and Sokobanja), and one in Bulgaria (Chiflik). This sampling covers the majority of the *A. maculatum* species distribution range (Appendix S1, Figure S1). During the typical flowering period of *A. maculatum* (April – May), we conducted field sampling each year between 2017 and 2019, visiting each site in at least two years during this timeframe.

2.3 – Common garden experiment sites

We selected the Swiss population of Neuchâtel and the French population Forêt du Gâvre (on the Atlantic fringe) as our common garden sites, since they were respectively dominated by *Psychoda phalaenoides* and *Psycha grisescens*. We conducted common garden experiments in Neuchâtel in 2018 and 2019, and in Forêt du Gâvre in 2019. These two sites experienced a relatively similar climate; over the course of our sampling in 2019, the mean temperature and humidity in Neuchâtel at the time of sampling were 15.8°C and 61% respectively, and in Forêt du Gâvre, the mean temperature and humidity were 17.2°C and 50% respectively. Five to ten *A. maculatum* individuals with unopened inflorescences were potted in soil from their native habitat, and then transplanted from all ten populations listed above to the two common garden sites. Inflorescences from Neuchâtel and Forêt du Gâvre were also reciprocally transplanted as part of this experiment. The total numbers of inflorescences sampled *in situ* and in both common garden sites are given in Appendix S1, Figure S1.

2.4 – Floral odour and pollinator sampling methods

During both the field surveys and common garden experiments, we collected dynamic headspace VOCs using identical methods, and analysed them using gas chromatography coupled to mass spectrometry (GC-MS; full details in Appendix S2 - Supplementary Methods). Briefly, we collected VOCs from *A. maculatum* inflorescences undergoing anthesis in the early evening on polydimethylsiloxane (PDMS) coated Twister® stir bars (Gerstel: Mülheim an der Ruhr, Germany), at an air flow rate of 200 mL min^{-1} for 30 minutes. Twisters® were kept on ice in sealed glass containers until GC-MS analyses, where volatiles were thermally desorbed and separated on a HP-5MS column. During the morning following VOC sampling, we collected all insects trapped within inflorescences and preserved them in 70% ethanol until identification (full identification methods detailed in Appendix S2 - Supplementary Methods).

2.5 – Characterizing and comparing floral odour variation across the range of A. maculatum

We compared total VOC emissions within and among populations of field-sampled inflorescences (*i.e.* VOCs sampled in their native habitats) using Bray-Curtis distance matrices, and visualized inter-individual variation using nonmetric multidimensional scaling (NMDS). Then, to test for a significant effect of population (fixed effect factor) on the entire VOC matrix, we performed pairwise permutational multivariate analysis of variance (PERMANOVA, Bray-Curtis distance, n = 999 permutations) with Bonferroni correction using the *adonis* function in the R v.3.6.1 (R Core Team 2019) package *vegan* (Oksanen et al. 2019).

2.6 – Characterizing temporal variation in pollinator community composition

We investigated inter-annual variation in pollinator community composition during our study (*i.e.* 2017 – 2019) by calculating Bray-Curtis distance matrices, comparing the mean quantities of pollinators trapped per inflorescence for all populations with *in situ* pollinator data collected in two or more years. We then visualized the result using nonmetric multidimensional scaling

(NMDS) ordinations. Next, we repeated this process to investigate shifts in the dominant pollinators trapped by *A. maculatum* over the past decade. Using Bray-Curtis distance matrices and NMDS ordinations, we compared the mean quantities of 1) Psychodidae species only, and 2) all insect families trapped per inflorescence in our study, with those collected approximately a decade ago (2006-2008; Espíndola et al. 2010).

2.7 – Identifying VOCs associated with species-specific pollinator attraction

Since we collected paired VOCs and pollinator data for each sampled inflorescence, we were able to correlate these two matrices and identify candidate compounds associated with the attraction of either *P. phalaenoides* or *P. grisescens.* To this aim, we used the Random Forest implementation in the R package *randomForest* (Liaw & Wiener 2002), with permutation importance enabled (*ntree* = 500, *mtry* = 8; optimized using the *tuneRF* function). We then calculated conditional feature contributions and identified combinations of compounds which had the greatest influence on the predictive strength of the classifier, using the Python package *TreeInterpreter* (Saabas 2019). As a measure of plasticity in these candidate compounds when flowering in different habitats (*i.e.* transplant effects), we calculated and plotted population standard scores of these compounds for both *in situ* and common garden samples. We then used Mann-Whitney *U*-tests to test whether populations with sufficient sample sizes ($n \geq 8$) emitted different quantities of candidate compounds when transplanted to common garden sites.

2.8 – Testing for local adaptation to pollinators using common garden experiments

We began by visualizing geographic patterns in mean pollinator attraction for each population *in situ*, and following transplants to both common garden sites. Then, following the 'local vs. foreign' definition of local adaptation (Kawecki & Ebert 2004), we tested the hypotheses that:

in the Neuchâtel common garden, transplanted *A. maculatum* inflorescences which attract *P. phalaenoides* in their native population should catch 1) more *P. phalaenoides* and/or 2) more pollinators in total than inflorescences which attract *P. grisescens* in their native population. We expected to observe the inverse result in the Forêt du Gâvre common garden (*i.e.* transplanted inflorescences that attract *P. grisescens* in their native population should perform better on average in Forêt du Gâvre). These expectations were tested using a two-way ANOVA on log+1 transformed pollinator counts, including 'native pollinator' (*i.e. P. phalaenoides*- or *P. grisescens*-dominated origin) and 'common garden location' (*i.e.* whether the common garden site was dominated by *P. phalaenoides* or *P. grisescens*) and their interaction as fixed factors. Here, a significant interaction would indicate local adaptation, which could be confirmed using contrasts comparing trait values between native and transplant sites. Finally, we analysed 'deme × habitat' interactions (Kawecki & Ebert 2004) for mean attraction rates of *P. phalaenoides*, *P. grisescens*, and all insects. Here, deme and habitat respectively referred to an *A. maculatum* population and its local pollinator community conditions. In this analysis, data were subset based on each inflorescence's native pollinator and common garden location, as described above.

3. RESULTS

3.1 – Floral odour is highly variable within and among A. maculatum populations

After filtering out compounds present in blank samples, we retained 18 *A. maculatum* floral VOCs present in relative abundances above 1% (Table 1). All of the major compounds we identified (e.g. indole, p-cresol, 2-heptanone, β-citronellene, and three unnamed sesquiterpenes) had been previously reported in studies of *A. maculatum* floral odour (Diaz & Kite 2002; Chartier et al. 2013; Marotz-Clausen et al. 2018). We observed substantial variation in floral odour both within (Figure 1) and among populations (Figure 2).

3.2 –Pollinator communities are variable at both annual & decadal scales

Our sampling allowed us to compare inter-annual (*i.e.* 2017/18 vs. 2019) variation in Psychodidae trapped by *A. maculatum* in six populations. The dominant Psychodidae species trapped by inflorescences appear to have shifted in three of these populations (Conteville, FR, Neuchâtel, CH, and Visuć, HRV) over this time period (Figure 3).

We also observed temporal shifts in the average abundances of trapped Psychodidae in several populations over the past decade (*i.e.* compared to Espíndola et al. 2010). The relative compositions of the complete pollinator communities trapped by *A. maculatum* appears to have shifted in five out of six populations where comparisons could be made; only Forêt du Gâvre remained consistent over the past decade (Appendix S1, Figure S2). We also observed apparent shifts in the dominant Psychodidae pollinator trapped within inflorescences in four out of six populations over the past decade, in Chaumont FR, Conteville FR, Gostilje SRB, and Visuć, HRV (Table 2 & Appendix S1, Figure S2).

3.3 – Low population-level VOC divergence coincides with temporally variable pollinators

PERMANOVA pairwise contrasts did not identify any pairs of populations with significant divergence in their proportional emissions of VOCs sampled in their native habitats, after correction for multiple testing. This lack of population-level differentiation in VOC blends is evident when visualizing Bray-Curtis similarities between field-sampled individuals (Figure 2), and appears to coincide with the temporal heterogeneity in pollinator communities identified in the previous section (see Figure 3 and Appendix S1, Figure S2).

3.4 – Arum maculatum populations typically are not locally adapted to specific pollinators

Through Random Forest analyses (Appendix S1, Figure S3), we identified one compound positively associated with *P. phalaenoides* attraction (β-humulene), and three compounds positively associated with *P. grisescens* attraction (unnamed sesquiterpenes RI 1470 and 1681, and α-selinene). After evaluating combined feature contributions within the Random Forest classifier, we found that its predictive strength was most strongly influenced by unnamed sesquiterpene (RI 1681) alone. Other strong combinations included unnamed sesquiterpene RI 1681 paired with unnamed sesquiterpene RI 1470 or α-selinene, as well as β-humulene alone, mirroring our initial results (full *Treeinterpreter* results in Appendix S1, Table S2).

The four candidate compounds we identified (β-humulene, unnamed sesquiterpenes RI 1470 and 1681, and α-selinene) were widely observed and not restricted to a specific region or pollinator background. Average emissions of these compounds remained relatively consistent between *in situ* collections and samples transplanted to Neuchâtel, though some appeared to increase after inflorescences were transplanted to Forêt du Gâvre (see Appendix S1, Figure S4). We only observed a significant transplant effect in one compound (an increase in unnamed sesquiterpene RI 1470) within one population, Neuchâtel (Mann-Whitney tests; full result in Appendix S1, Table S3).

In both common garden sites, we observed that transplanted inflorescences typically attracted the dominant local pollinator species as efficiently as native inflorescences (Figure 1). While common garden location had a significant effect on the quantity of *P. phalaenoides* and *P. grisescens* caught by *A. maculatum* (*i.e.* the two transplant sites were dominated by different Psychodidae species), no native pollinator × common garden location interaction effect was observed (2-way ANOVA, $Pr(>F) > 0.05$; full results in Appendix S1, Table S4). Together, these results suggest that *A. maculatum* populations are generally not locally adapted to a single

pollinator species (Figure 4), with notable exceptions in Forêt du Gâvre and the two Serbian populations Gostilje and Sokobanja, which will be discussed below.

While we do not find general indications of populations being locally adapted to specific pollinators, not all inflorescences were equally attractive to all pollinator species. One population (Forêt du Gâvre) continued to exclusively attract their native pollinator *P. grisescens* when transplanted to the Neuchâtel common garden (see Figure 1). Remarkably, this exclusive attraction of *P. grisescens* was maintained even though *P. phalaenoides* was also present (and trapped by inflorescences from other populations) at the time when inflorescences from Forêt du Gâvre opened (see Appendix S1, Figure S5a). Additionally, transplanted inflorescences occasionally attracted the 'non-dominant' Psychodidae species in both common garden sites, and a third *Psychoda* species (*Psychoda trinodulosa*) was also identified within inflorescences in Croatia and Serbia during our field surveys. *Psychoda trinodulosa* was also observed in the Neuchâtel common garden, but not in Forêt du Gâvre; inflorescences from both Serbian populations (Gostilje and Sokobanja) continued to occasionally attract *P. trinodulosa* when transplanted to the Neuchâtel common garden (Figure 1).

4. DISCUSSION

In this study, we identified substantial within-and among- population variation in *A. maculatum* floral odour (Figure 1 and Figure 2) and shifts in pollinator community composition in several populations at both the inter-annual (Figure 3) and decadal scales (Appendix S1, Figure S2). Although some *A. maculatum* populations continued to attract their native pollinators in transplant sites (e.g. Forêt du Gâvre and Serbian populations), most populations did not exclusively attract *P. phalaenoides* or *P. grisescens* when transplanted to 'foreign' pollinator backgrounds (Figure 4). Since this result does not appear to be due to transplant effects /

phenotypic plasticity (Appendix S1, Table S3), this leaves temporally heterogeneous pollinator communities as a potential mechanism underlying the maintenance of high within-population variation in floral odour.

4.1 – The influence of temporally variable pollinators on A. maculatum floral odour

Overall, it appears that local pollinator availability at the time of anthesis plays a key role in the quantity and composition of pollinators trapped within *A. maculatum* inflorescences. Previous studies on *A. maculatum* pollination in England (Diaz & Kite 2002) and France (Chartier et al. 2013) arrived at a similar conclusion. The difference in sex ratios, particularly that no male *P. phalaenoides* or *P. trinodulosa* are attracted, may be due to differences in copulation timing among *Psychoda* species (*i.e.* immediately after adult emergence), and parthenogenetic reproduction (F. Vaillant, cited in Albre et al. 2003), or brood-site specific VOCs which are only attractive to females of these two species. While we were unable to reliably collect data on background pollinator communities as part of our sampling design, Diaz & Kite (2002) found that Psychodidae species caught on sticky traps mirrored those found within *A. maculatum* and *A. italicum* inflorescences. Therefore, our data should still allow us to infer whether the patterns in pollinator attraction we observed within each population were a consequence of balancing selection or local adaptation.

Balancing selection may maintain variation within populations through several selective regimes, including relaxed selection, negative frequency-dependent selection due to pollinator learning, or environmental heterogeneity (Delph & Kelly 2013). While relaxed selection on floral odour has been observed in some angiosperms (Salzmann et al. 2007), this is unlikely to be the case for deceptively pollinated *A. maculatum*, given that pollinator attraction is driven by VOC emissions alone (Dormer 1960; Lack & Diaz 1991). Pollinator learning has been

shown to maintain polymorphism in floral colour (Gigord et al. 2001) and odour (Ayasse et al. 2000), but this is also less likely to occur in the case of *A. maculatum*. Psychodidae likely experience weak and inconsistent selective pressures caused by deception, due to their large population sizes and short generation times (Prime 1960; Lachmann et al. 2000), and potentially sex-specific attraction in some cases. Araceae also exploit pre-existing VOC detection abilities in their pollinators (Schiestl & Dötterl 2012), which likely contributes to their persistent inability to distinguish deceptive inflorescences (Renner 2006). This leaves temporal heterogeneity in pollinator communities as the mechanism most likely contributing to the maintenance of diverse floral odour bouquets in *A. maculatum*.

In this study, we demonstrated that pollinator communities can vary within populations over relatively short time periods (*i.e.* over the years- to decade-scale) across most of the range of *A. maculatum*. Temporally heterogeneous pollinators have long been known to influence floral traits (Schemske & Horvitz 1989), but to our knowledge, have not been investigated as a mechanism underlying floral odour variation. Further research on temporal variation in pollinators may provide clarity in other cases where floral trait variation in deceptively pollinated species cannot be explained by pollinator learning (e.g. Pellegrino et al. 2005; Jersáková et al. 2006). Temporally variable pollinators may have also contributed to the finding that, contrary to expectations, deceptively pollinated species generally do not maintain more variable floral odours than rewarding species (Ackerman et al. 2011; Delle-Vedove et al. 2017).

Currently, it is not known whether the phenologies of *P. phalaenoides* and *P. grisescens* are influenced by environmental variation; our data indicate that at least in Forêt du Gâvre, *P. grisescens* emerges slightly earlier than *P. phalaenoides* (Appendix S1, Figure S5b). A recent environmental DNA survey of cow dung (Sigsgaard et al. 2020) also identified *P.*

phalaenoides, *P. trinodulosa*, and *P. grisescens* all within a single site in Mols Bjerge, Denmark. At the time the sample was taken (June 2019; *i.e.* later than our field surveys), *P. phalaenoides* overwhelmingly predominated over all other insects in terms of read counts (*i.e.* abundance). Taken together, these results corroborate two key points: all of the major Psychodidae pollinators of *A. maculatum* are present across the range of *A. maculatum* (Ježek et al. 2018), and their relative abundances may vary temporally over the course of the flowering period.

If environmental variation influences pollinator phenology, then plasticity in floral odour based on the same environmental cues might enhance pollinator attraction. With one exception in a single population (*i.e.* unnamed sesquiterpene RI 1470 emitted by inflorescences from Neuchâtel), we did not observe significant shifts in the four candidate compounds linked to species-specific pollinator attraction following transplants to common gardens (*i.e.* phenotypic plasticity). However, as we transplanted mature inflorescences, some environmental effects may have already been in place prior to transplanting (Wund 2012). Growing *A. maculatum* for several generations in common garden sites would give a more accurate result and eliminate maternal effects, but this was not feasible within the scope of our study, as *A. maculatum* requires at least two years (and sometimes longer) from germination to flowering (Brown 2000). Given the central role of VOCs in *A. maculatum* pollination (Kite 1995; Kite et al, 1998), it is unlikely that the high variation in floral odour we observed is the result of plasticity alone. Further research tracking the VOC emissions of the same individual over multiple flowering events would provide greater resolution into environmental influences on floral odour variation.

Since *A. maculatum* are rhizomatous, variation may also persist within populations for longer periods of time compared to annual plants. Gene flow between populations may also contribute to the maintenance of floral odour variation within populations. Pollen dispersal by Psychodidae is likely limited due to their poor flying abilities and short adult lifespans (Lack & Diaz 1991), but seed dispersal by frugivorous birds (Snow & Snow 1988) could lead to gene flow among nearby populations. While there appears to be a strong barrier to gene flow between populations from north/central Europe, and from Italy and the Balkans (Espíndola & Alvarez 2011), our data suggest that floral odour variation is widely maintained across this barrier. This pattern suggests that — at least regionally — balancing selection is at work, possibly in association with phenotypic plasticity and/or local adaptation in populations with consistent pollinator communities.

4.2 – The influence of spatially variable pollinators on A. maculatum floral odour

The results from our Europe-wide transplant and common garden experiment do not support the hypothesis that all *A. maculatum* populations are (locally) adapted to attract exclusively *P. phalaenoides* or *P. grisescens*. This contrasts with the patterns found in other wide-ranging deceptively pollinated species such as the sexually deceptive orchids *Ophrys sphegodes* (Breitkopf et al. 2013) and *Ophrys insectifera* (Triponez et al. 2013), but aligns with the results of a previous reciprocal transplant experiment between two *A. maculatum* populations in France (Chartier et al. 2013). However, we found that the Forêt du Gâvre population in coastal NW France appears to have lost the ability to attract *P. phalaenoides*, possibly due to local adaptation and/or genetic drift; the latter process is known to occur at the limits of species ranges (Geber 2011; Gould et al. 2013). Interestingly, Forêt du Gâvre is also the only population in this study with little recorded inter-annual and decade-scale variation in pollinator communities. It is therefore possible that the pollinator attraction patterns we

observed are a result of selective pressures imposed by the likely stable population of *P. grisescens* in this site. Further transplants of Forêt du Gâvre inflorescences to additional populations are needed to test this hypothesis.

Our results also do not exclude the possibility that individual VOCs are differentially attractive to certain Psychodidae species. First, we identified four sesquiterpenes that were able to predict the attraction of *P. phalaenoides* (β-humulene) and *P. grisescens* (unidentified sesquiterpenes RI 1470 and 1681, and α-selinene) through Random Forest analyses. Additionally, both Serbian populations in this study (Gostilje and Sokobanja) attracted a third Psychodidae species (*P. trinodulosa*) *in situ* and following transplants to the Neuchâtel common garden, suggesting that there may also be VOCs related to species-specific attraction of *P. trinodulosa*. These patterns suggest that specific compounds emitted by inflorescences from Serbia may also be differentially attractive to *P. trinodulosa*. However, this species was too infrequently observed in our study to make any firm conclusions; further studies of these populations are needed to address this question.

In this study, the VOCs typically associated with Psychodidae brood sites (e.g. indole, p-cresol, and 2-heptanone) were not correlated with species-specific pollinator attraction. A recent study by Gfrerer et al. (2021) also found that variation in the aforementioned foetid-smelling VOCs were not among those which significantly influenced fruit sets in *A. maculatum*. Given that blends of indole, p-cresol, and 2-heptanone are known to be attractive to *Psychoda* species (Kite et al. 1998), these compounds appear to be generally attractive to all species. However, blends of VOCs (e.g. with specific sesquiterpenes) or the ratios at which they are emitted may be differentially attractive. Behavioural assays, where different pollinator species are presented with several *A. maculatum* inflorescences or individual VOCs, would be useful in identifying which compounds or blends elicit general or species-specific responses. Similarly, Gas

Chromatography-Electroantennography (GC-EAD; Cork et al. 1990) could be used to identify all VOCs which elicit a physiological response in different pollinator species.. The *A. maculatum* populations with unique patterns in pollinator attraction identified in this study may be useful targets for future research testing whether selection is acting on specific compounds (e.g. Strensmyr et al. 2002; Urru et al. 2010), or on "super-attractive mixtures" (e.g. in *A. palaestinum*; Stökl et al. 2010). In either case, our results still allow us to conclude that variable floral odour blends are effective in the specialized

Conclusion

To date, almost all studies on floral odour and pollinator variation have been carried out at a single timepoint — possibly contributing to the numerous cases where floral odour diversity appears to exceed pollinator diversity (Delle-Vedove et al. 2017). As evidenced by the extensive literature on local adaptation in plants (Leimu & Fischer 2008), spatially varying selection is an undoubtedly important driver of floral odour divergence, and appears to have influenced a few *A. maculatum* populations (e.g. Forêt du Gâvre) as well. However, the variable floral odour bouquets emitted by *A. maculatum* may also represent an important adaptation to temporally heterogeneous pollinator communities, if the pollinator shifts we observed have been occurring over longer periods of time. In closing, we advocate for increased attention on the temporal dimension of pollinator-mediated selection. By simultaneously studying spatial and temporal variation in selection, we may further our understanding of how and why flowering plants maintain high diversity in key functional traits such as floral odour.

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TABLES

Table 1. Average proportional emissions (M) of volatile organic compounds (+/- SD) emitted by north/ central populations of *Arum maculatum*. Compounds representing more than 5% of the average total blend are highlighted in bold.

		Forêt du Gâvre, France (n = 14)			**Conteville, France** (n = 10)			**Chaumont, France** (n = 8)			**Neuchâtel, Switz-erland** (n = 21)			**Cortaillod, Switz-erland** (n = 29)		
VOC	**RI**	M	SD	Freq	M	SD	Freq	M	SD	Freq	M	SD	Freq	M	SD	Freq
2-heptanone	891	1.09	1.69	50.0	**7.74**	**16.54**	**40.0**	4.68	10.37	25.0	0.14	0.29	28.6	1.12	2.01	41.4
β-citronellene	943	3.58	6.02	64.3	0.02	0.07	10.0	2.25	2.28	75.0	**6.32**	**7.17**	**81.0**	3.39	3.74	82.8
cis β-ocimene	1037	0.10	0.37	7.1	0.00	0.00	0.0	0.02	0.05	12.5	0.32	0.75	33.3	0.51	1.02	27.6
p-cresol	1076	4.04	7.54	42.9	**5.71**	**10.15**	**30.0**	0.04	0.12	12.5	0.79	2.11	14.3	0.00	0.00	0.0
indole	1289	**22.50**	**15.45**	**100**	**19.92**	**20.11**	**90.0**	**15.23**	**15.61**	**87.5**	**39.02**	**30.26**	**95.2**	**46.26**	**26.81**	**96.6**
skatole	1383	1.89	2.84	50.0	1.44	2.13	50.0	4.21	2.28	87.5	1.59	1.84	57.1	1.62	1.41	69.0
α-copaene	1374	0.02	0.04	28.6	0.01	0.02	20.0	0.25	0.53	25.0	0.02	0.07	14.3	0.48	2.06	20.7
Z-caryophyllene	1405	**9.26**	**11.07**	**71.4**	1.87	4.44	30.0	2.11	2.00	62.5	**8.39**	**7.52**	**85.7**	3.94	4.74	75.9
β-caryophyllene	1416	1.48	2.14	50.0	**7.87**	**9.84**	**70.0**	1.92	2.30	62.5	1.37	1.93	47.6	2.82	5.68	72.4
α-humulene	1452	**5.15**	**4.43**	**71.4**	**17.55**	**22.93**	**70.0**	**11.32**	**2.66**	**100**	**5.82**	**5.44**	**76.2**	3.87	3.10	72.4
alloaromadendrene	1459	4.66	3.81	85.7	3.96	4.53	60.0	**7.02**	**4.26**	**87.5**	**5.62**	**4.41**	**85.7**	**8.09**	**9.94**	**82.8**
β-humulene	1473	**5.46**	**7.02**	**64.3**	1.24	1.95	40.0	**10.13**	**5.87**	**87.5**	4.97	6.45	85.7	2.72	1.91	82.8
α-selinene	1491	2.06	3.68	35.7	0.35	1.12	10.0	3.97	4.52	62.5	1.66	3.43	28.6	0.43	1.18	17.2
bicyclogermacrene	1493	0.58	1.02	42.9	0.87	2.75	10.0	1.18	1.70	50.0	2.64	4.07	57.1	**6.46**	**9.51**	**75.9**
d-cadinene	1520	0.48	1.09	35.7	1.63	4.66	20.0	**9.59**	**12.96**	**100**	3.75	6.47	57.1	1.55	2.14	58.6
Unnamed sesqui.	1404	0.63	1.21	42.9	0.04	0.14	10.0	0.20	0.37	37.5	4.94	10.48	57.1	2.49	6.65	37.9
Unnamed sesqui.	1470	4.62	6.00	71.4	2.17	3.98	30.0	**11.17**	**5.59**	**100**	3.48	3.27	76.2	3.96	2.82	79.3
Unnamed sesqui.	1681^	**32.39**	**20.75**	**100**	**27.61**	**17.68**	**80.0**	**14.71**	**8.88**	**100**	**9.15**	**14.36**	**81.0**	**10.29**	**10.45**	**75.9**

Table 1 (cont). Average proportional emissions (M) of volatile organic compounds (+/- SD) emitted by southern (Italy) and eastern (Balkan) populations of *Arum maculatum*. Compounds representing more than 5% of the average total blend are highlighted in bold.

		Montese, Italy (n = 13)			**Visuć, Croatia** (n = 6)			**Gostilje, Serbia** (n = 19)			**Sokobanja, Serbia** (n = 11)			**Chiflik, Bulgaria** (n = 3)		
VOC	**RI**	M	SD	Freq	M	SD	Freq	M	SD	Freq	M	SD	Freq	M	SD	Freq
2-heptanone	891	**8.28**	**14.17**	**61.5**	**29.36**	**27.79**	**100**	0.00	0.00	0.0	0.00	0.00	0.0	0.00	0.00	0.0
β-citronellene	943	3.26	5.49	69.2	**8.77**	**2.18**	**100**	0.65	2.12	21.1	0.05	0.15	9.1	0.00	0.00	0.0
cis β-ocimene	1037	0.21	0.40	30.8	0.32	0.79	16.7	0.01	0.04	5.3	0.00	0.00	0.0	0.00	0.00	0.0
p-cresol	1076	**7.58**	**11.99**	**61.5**	4.34	5.81	66.7	0.00	0.00	0.0	0.00	0.00	0.0	2.85	4.94	33.3
indole	1289	**22.38**	**16.81**	**92.3**	**7.99**	**9.73**	**83.3**	**55.41**	**26.79**	**100**	**43.41**	**37.98**	**90.9**	**54.24**	**27.05**	**100**
skatole	1383	1.46	1.49	61.5	1.07	1.10	66.7	4.34	5.80	73.7	2.92	3.19	63.6	1.84	0.45	100
α-copaene	1374	0.32	0.64	46.2	0.10	0.20	50.0	1.61	4.51	68.4	4.46	10.94	72.7	2.52	2.65	66.7
Z-caryophyllene	1405	**12.78**	**14.31**	**69.2**	**22.25**	**20.66**	**83.3**	4.65	6.29	63.2	**9.88**	**19.53**	**45.5**	**6.90**	**3.86**	**100**
β-caryophyllene	1416	0.88	1.20	38.5	1.96	2.50	50.0	0.54	2.06	15.8	0.73	2.34	18.2	0.00	0.00	0.0
α-humulene	1452	6.25	8.75	76.9	**6.11**	**5.24**	**100**	**6.91**	**10.62**	**57.9**	1.81	3.34	27.3	0.70	1.21	33.3
alloaromadendrene	1459	2.89	2.70	69.2	2.04	1.65	83.3	1.86	3.22	36.8	4.71	5.97	54.5	0.73	1.26	33.3
β-humulene	1473	**8.07**	**13.22**	**100**	0.75	1.09	50.0	1.54	2.77	42.1	2.64	4.09	36.4	0.67	1.16	33.3
α-selinene	1491	0.88	1.55	30.8	0.00	0.00	0.0	1.38	3.12	31.6	0.47	1.11	18.2	0.00	0.00	0.0
bicyclogermacrene	1493	0.43	0.69	38.5	0.00	0.00	0.0	1.02	1.60	47.4	0.97	2.18	27.3	**6.95**	**12.04**	**33.3**
d-cadinene	1520	1.36	2.55	46.2	0.62	0.96	33.3	2.36	8.28	21.1	0.28	0.76	18.2	0.00	0.00	0.0
Unnamed sesqui.	1404	0.47	0.87	30.8	0.00	0.00	0.0	0.99	2.61	42.1	0.74	1.43	27.3	0.00	0.00	0.0
Unnamed sesqui.	1470	3.35	3.61	69.2	4.06	2.17	100	2.99	5.27	52.6	4.97	7.83	36.4	4.00	1.71	100
Unnamed sesqui.	1681^	**19.15**	**18.28**	**84.6**	**10.23**	**12.36**	**100**	**13.73**	**13.14**	**84.2**	**21.96**	**20.18**	**81.8**	**18.60**	**15.25**	**100**

RI = Kovats retention index (^ = RI 1716 in Diaz & Kite 2002)
Freq = proportion of chromatograms (%) in which the compound was identified in a given population.

Table 2. Mean *in-situ* abundances of *Psychoda phalaenoides* and *Psycha grisescens* individuals trapped per *Arum maculatum* inflorescence between 2006-2008 (data from Espíndola et al. 2010), and in our field surveys between 2017-2019. The number of individual inflorescences sampled *in situ* are indicated in parentheses. NAs indicate that no inflorescences were open yet at the time populations were visited.

Poll. Species / Year / **Population**	***P. phalaenoides* (2008)**	***P. grisescens* (2008)**	***P. phalaenoides* (2019)**	***P. grisescens* (2019)**
Fôret du Gavre (France)	0 (5)	6.4 (5)	0 (16)	25.7 (16)
Conteville (France)	47.6 (8)	2.3 (8)	0.4 (14)	1.3 (14)
Chaumont (France)	1.6 (6)	0.5 (6)	0 (7)	0.6 (7)
Lausanne/Neuchâtel (Switzerland)	3.0 (2)	0 (2)	2.0 (44)	0.6 (44)
Montese (Italy)	0 (5)	2 (5)	NA	NA
Visuć (Croatia)	0.1 (7)	0.6 (7)	1.3 (6)	3.0 (6)
Gostilje (Serbia)	0 (5)	2.4 (5)	0.4 (18)	0 (18)
Sokobanja (Serbia)	0 (5)	0.2 (5)	0 (21)	0.1 (21)
Chiflik (Bulgaria)	0.4 (7)	4.3 (7)	NA	NA

FIGURE CAPTIONS

Figure 1. Europe-wide variation in floral volatile organic compounds (VOCs) and pollinator attraction of *Arum maculatum*. Stacked barplots present the relative quantities of VOCs in two terpene classes (mono- and sesquiterpenes) and four dung-mimicking VOCs emitted by individual inflorescences. Pie charts indicate the composition of Psychodidae trapped by *A. maculatum* inflorescences during field surveys (on map), and following transplants to two common garden sites (below map), scaled to represent mean quantities of Psychodidae per plant. Dotted lines link each field result with corresponding transplant results. *Note: empty chart = no Psychodidae attracted during our surveys*.

Figure 2. A) Multivariate representation of *Arum maculatum* floral volatile organic compound (VOC) emissions, using nonmetric multidimensional scaling (NMDS) of Bray-Curtis distances between individuals. Points and ellipses are coloured according to individuals' population of origin. B) Circle plot visualizing the correlations between all VOCs and the NMDS axes.

Figure 3. Interannual changes (2017/18 – 2019) in the average composition of Psychodidae pollinators trapped by six *Arum maculatum* populations. [NMDS; Bray-Curtis Distance; overall stress = 0.005]

Figure 4. Pollinator attraction in the reciprocal transplant experiment. Plots show the mean (+/- SE) numbers of A) *Psychoda phalaenoides*, B) *Psycha grisescens*, and C) all insects trapped by inflorescences, transplanted from either *P. phalaenoides*-dominated populations (filled circles) or *P. grisescens*-dominated populations (hollow circles). No deme × habitat interactions (indicative of local adaptation) were identified.

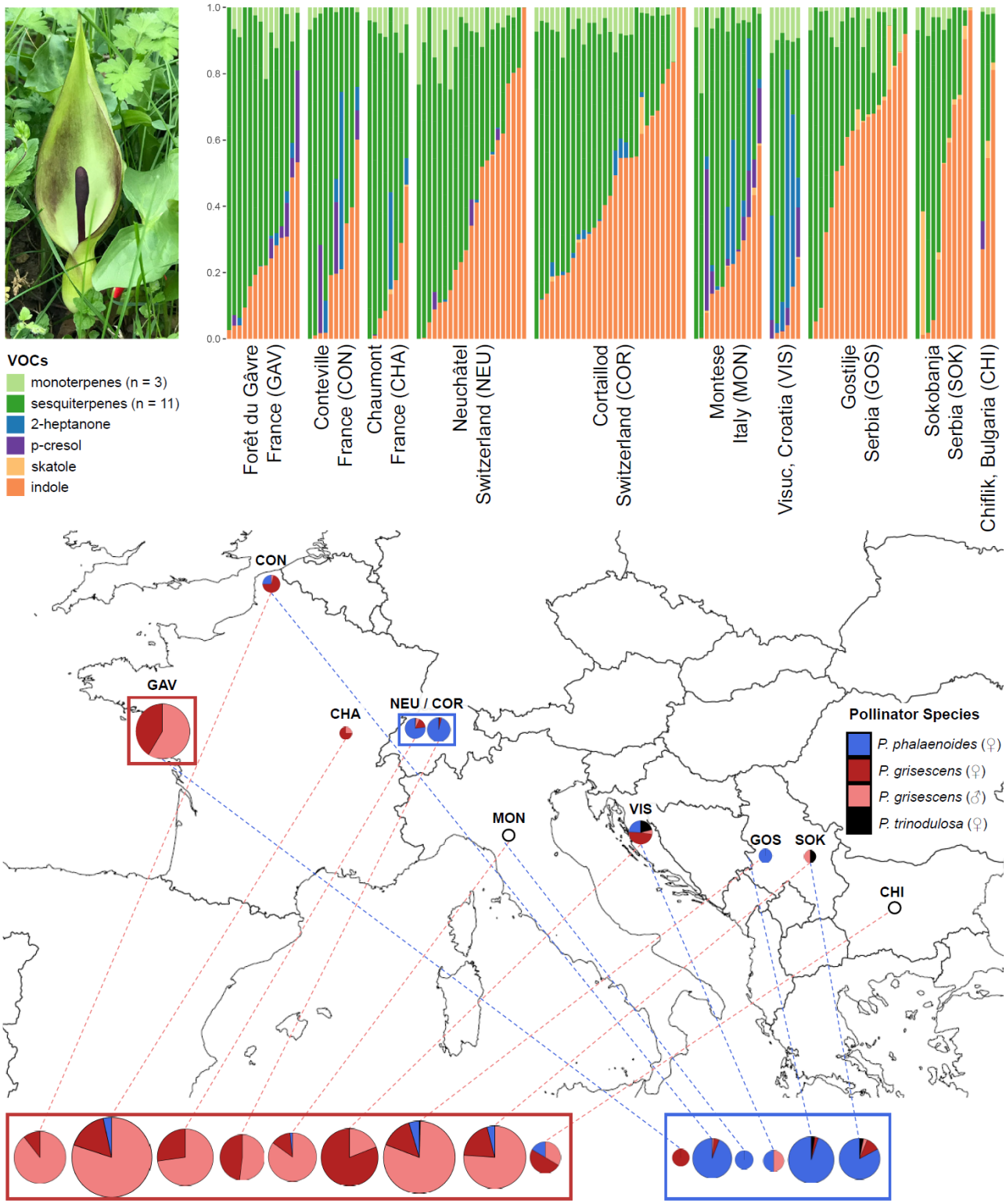
1.0
0.8
0.6
0.4
0.2
0.0
VOCs
monoterpenes (n = 3)
sesquiterpenes (n = 11)
2-heptanone
p-cresol
skatole
indole
Forêt du Gâvre France (GAV)
Conteville France (CON)
Chaumont France (CHA)
Neuchâtel Switzerland (NEU)
Cortaillod Switzerland (COR)
Montese Italy (MON)
Visuc, Croatia (VIS)
Gostilje Serbia (GOS)
Sokobanja Serbia (SOK)
Chiflik, Bulgaria (CHI)
CON
GAV
CHA
NEU / COR
MON
VIS
GOS
SOK
CHI
Pollinator Species
P. phalaenoides (♀)
P. grisescens (♀)
P. grisescens (♂)
P. trinodulosa (♀)

Fig.1

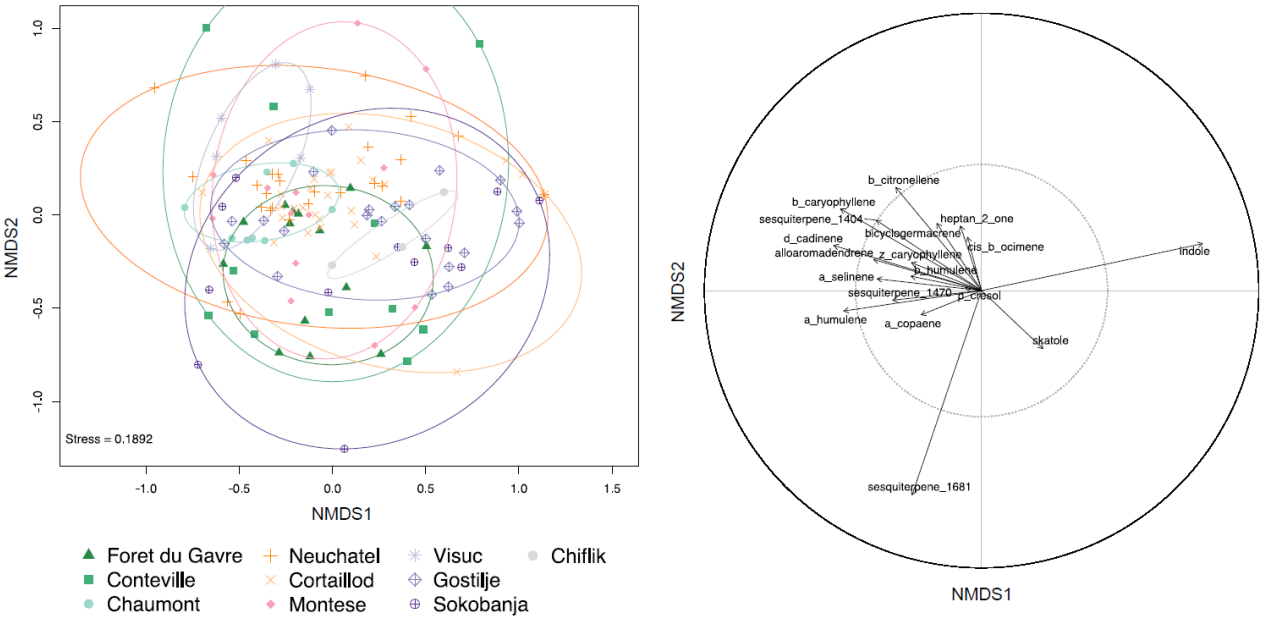
NMDS2
NMDS1
Stress = 0.1892
Foret du Gavre
Conteville
Chaumont
Neuchatel
Cortaillod
Montese
Visuc
Gostilje
Sokobanja
Chiflik
b_citronellene
b_caryophyllene
sesquiterpene_1404
heptan_2_one
d_cadinene
bicyclogermacrene
cis_b_ocimene
alloaromadendrene
z_caryophyllene
indole
a_selinene
b_humulene
sesquiterpene_1470
p_cresol
a_humulene
a_copaene
skatole
sesquiterpene_1681

Fig.2

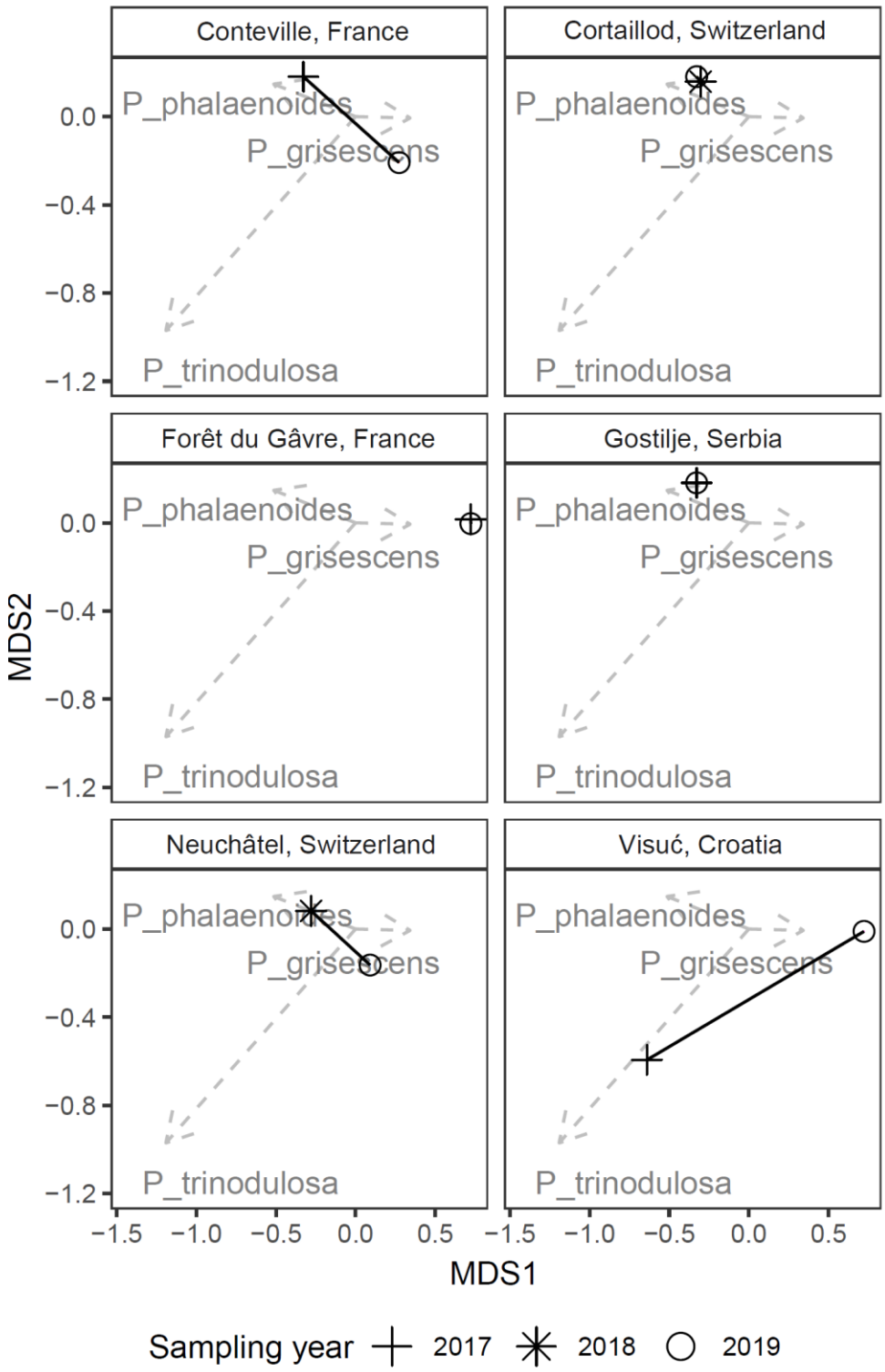
Conteville, France
Cortaillod, Switzerland
Forêt du Gâvre, France
Gostilje, Serbia
Neuchâtel, Switzerland
Visuć, Croatia
P_phalaenoides
P_grisescens
P_trinodulosa
MDS2
MDS1
0.0
−0.4
−0.8
−1.2
−1.5
−1.0
−0.5
0.0
0.5
Sampling year
2017
2018
2019

Fig.3

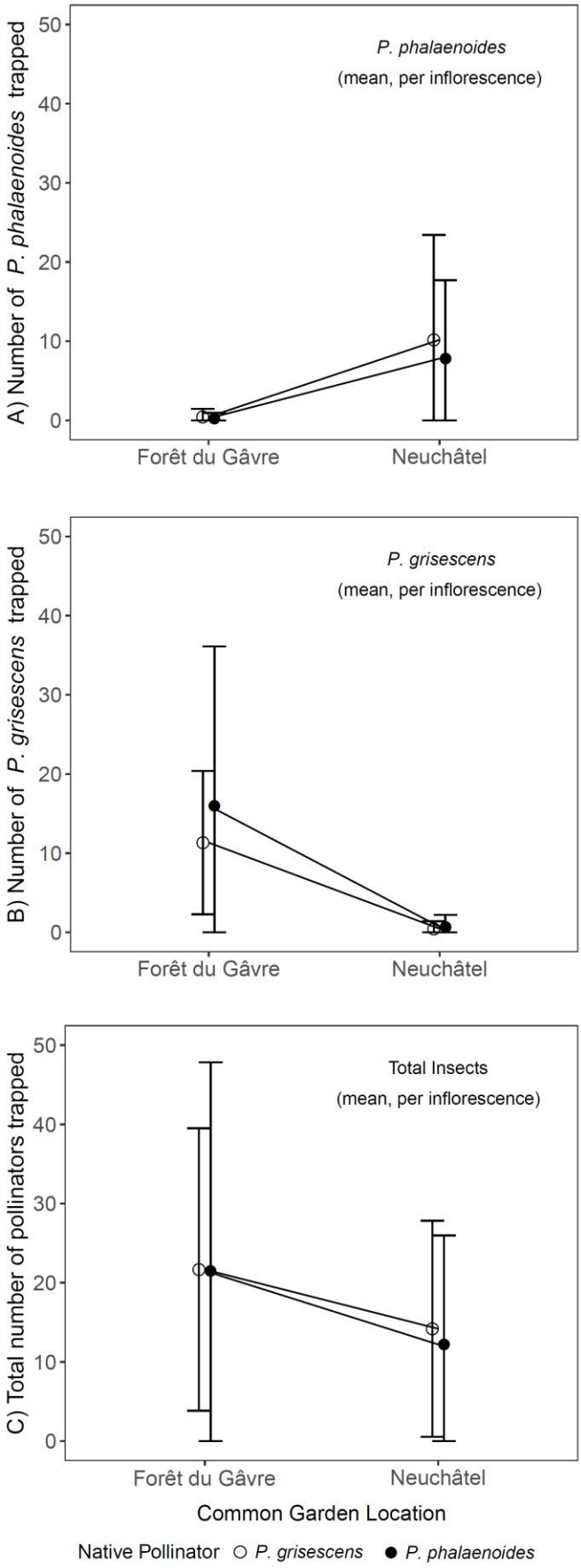
A) Number of P. phalaenoides trapped
P. phalaenoides
(mean, per inflorescence)
B) Number of P. grisescens trapped
P. grisescens
(mean, per inflorescence)
C) Total number of pollinators trapped
Total Insects
(mean, per inflorescence)
0
10
20
30
40
50
Forêt du Gâvre
Neuchâtel
Common Garden Location
Native Pollinator ○ P. grisescens ● P. phalaenoides

Fig.4