Annals of Botany **121**: 711–721, 2018
doi:10.1093/aob/mcx203, available online at www.academic.oup.com/aob

ANNALS OF BOTANY
Founded 1887

Diel pattern of floral scent emission matches the relative importance of diurnal and nocturnal pollinators in populations of *Gymnadenia conopsea*

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Received: 17 May 2017 Returned for revision: 23 October 2017 Editorial decision: 6 December 2017 Accepted: 9 January 2018
Published electronically 18 January 2018

• **Background and Aims** Floral scent is considered an integral component of pollination syndromes, and its composition and timing of emission are thus expected to match the main pollinator type and time of activity. While floral scent differences among plant species with different pollination systems can be striking, studies on intraspecific variation are sparse, which limits our understanding of the role of pollinators in driving scent divergence.
• **Methods** Here, we used dynamic headspace sampling to quantify floral scent emission and composition during the day and at night in the natural habitat of six Scandinavian populations of the fragrant orchid *Gymnadenia conopsea*. We tested whether diel scent emission and composition match pollinator type by comparing four populations in southern Sweden, where nocturnal pollinators are more important for plant reproductive success than are diurnal pollinators, with two populations in central Norway, where the opposite is true. To determine to what extent scent patterns quantified in the field reflected plasticity, we also measured scent emission in a common growth chamber environment.
• **Key Results** Both scent composition and emission rates differed markedly between day and night, but only the latter varied significantly among populations. The increase in scent emission rate at night was considerably stronger in the Swedish populations compared with the Norwegian populations. These patterns persisted when plants were transferred to a common environment, suggesting a genetic underpinning of the scent variation.
• **Conclusions** The results are consistent with a scenario where spatial variation in relative importance of nocturnal and diurnal pollinators has resulted in selection for different scent emission rhythms. Our study highlights the importance of adding a characterization of diel variation of scent emission rates to comparative studies of floral scent, which so far have often focused on scent composition only.

Key words: Diel variation, diurnal and nocturnal pollination, floral evolution, floral scent, geographic variation, *Gymnadenia conopsea* (fragrant orchid), plasticity, population differentiation, scent emission rate, scent rhythm, semi-generalized pollination, spatial variation.

INTRODUCTION

Plant–pollinator interactions are mediated via the emission of sensory signals by flowers and their reception by pollinators. Whereas pollinator-driven adaptation of visual cues and floral morphologies provide some of the best examples of natural selection (e.g. Bradshaw and Schemske, 2003; Whittall and Hodges, 2007), the evolution and diversification of floral scent is less understood (Raguso, 2008; Junker and Parachnowitsch, 2015). For instance, studies documenting phenotypic selection on scent are few and recent (Schiestl *et al.*, 2010; Ehrlén *et al.*, 2012; Parachnowitsch *et al.*, 2012; Gross *et al.*, 2016), and, although floral scent is predicted to be under pollinator-mediated selection, the selective agents are still largely unidentified, as is the importance of floral scent evolution for plant diversification.

Floral scents are very diverse among species (Knudsen *et al.*, 2006), and two observations suggest that this diversity is the result of pollinator-driven evolution. First, scent profiles often converge across unrelated plant species with similar pollination modes (Fenster *et al.*, 2004; Dobson, 2006; Junker and Parachnowitsch, 2015), such as the association between sulphur-containing compounds and bat pollination (Knudsen and Tollsten, 1995) and between the dominance of aromatic compounds and lepidopteran pollination (Knudsen and Tollsten, 1993; Andersson *et al.*, 2002). Secondly, floral scent can be differentiated even among closely related taxa when these differ in pollination systems, such as, for example, lepidopteran vs. beefly pollination in *Narcissus* species (Dobson *et al.*, 1997) or bee vs. hummingbird pollination in two *Mimulus* species (Byers *et al.*, 2014), suggesting that differences in the dominating functional group of pollinators drive divergence in floral scent.

If interspecific floral scent variation is the result of pollinator-mediated selection, we should also expect among-population scent variation when plant species vary in the dominating pollinators across their distributional range. Most studies examining among-population variation in scent composition come from specialized systems, where pollinators belong to the same functional group (*sensu* Fenster *et al.*, 2004). Some display substantial scent variation consistent with spatial variation in pollinator species (Breitkopf *et al.*, 2013) or pollinator

physiology (Suinyuy *et al.*, 2015), while other specialized systems show little variation in floral scent among populations pollinated by different species (Dötterl *et al.*, 2005; Svensson *et al.*, 2005; Ibanez *et al.*, 2010). For plant species with more generalized pollination systems, studies are few, and here, also, results are mixed. The cactus *Echinopsis ancistrophora* ssp. *ancistrophora* shows surprisingly little variation in floral scent composition among populations pollinated by either hawkmoths or bees (Schlumpberger and Raguso, 2008), whereas scent in the orchid *Gymnadenia odoratissima* was found to vary between high- and low-altitude populations whose pollinator assemblies differed most markedly in the presence or absence of empidid flies (Sun *et al.*, 2014; Gross *et al.*, 2016). Clearly, there is a need for additional studies that investigate how and to what extent floral scent variation is associated with variation in pollinator communities.

Populations may also differ in the diel rhythm of scent emission. If floral scent production is costly, as suggested by the reduction in floral scent in evolutionary transitions to self-pollination or bird pollination (Doubleday *et al.*, 2013; Amrad *et al.*, 2016; Sas *et al.*, 2016), the timing of scent emission should match the period of activity of the pollinators targeted by this signal, to avoid wasting resources or attracting antagonists. Indeed, diel variation in total floral scent emissions matching pollinator peak activity seems common in both diurnally (Theis *et al.*, 2007; Borges *et al.*, 2013; Friberg *et al.*, 2014; Burdon *et al.*, 2015) and nocturnally pollinated species (Effmert *et al.*, 2005; Dötterl *et al.*, 2005; Hoballah *et al.*, 2005). For species pollinated both during the day and at night, the evidence is mixed: scent emissions peaked at the expected period given data on diurnal and nocturnal pollinator visits or the relative contribution of the two types of pollinators to fitness in some species (Dötterl *et al.*, 2012; Jürgens *et al.*, 2014; van der Niet *et al.*, 2015), but not in others (Morinaga *et al.*, 2008; van der Niet *et al.*, 2015; Prieto-Benítez *et al.*, 2016), suggesting that other factors also influence the evolution of floral scent emission rates. To our knowledge, no previous study has examined diel scent emission in a system with among-population variation in relative abundance of diurnal and nocturnal pollinators. This should be particularly informative for testing the hypothesis that diel rhythms in floral scent emission rates can diverge in response to differences in dominating pollinators.

Scent emission rates and composition can be influenced by environmental factors, including temperature (e.g. Hansted *et al.*, 1994; Jakobsen and Olsen, 1994; Farré-Armengol *et al.*, 2014; Friberg *et al.*, 2014), humidity (Jakobsen and Olsen, 1994; Friberg *et al.*, 2014), light (Jakobsen and Olsen, 1994; Friberg *et al.*, 2014) and nutrient availability (Majetic *et al.*, 2017). This makes it is difficult to draw strong inferences about the importance of plant–pollinator interactions for the evolution of floral scent variation from field data alone (Majetic *et al.*, 2009). Thus, there is a need to disentangle innate and environmentally induced causes of floral scent variation among populations by collecting data on scent variation in a common environment.

In this study, we quantify scent composition and diel emission rhythms in six Scandinavian populations of the fragrant orchid *Gymnadenia conopsea*, two in central Norway and four in southern Sweden, that vary in the relative abundance of diurnal and nocturnal pollinators and in the species composition of these pollinator categories. A combination of pollinator observations and selective pollinator exclusion experiments indicates that diurnal pollination contributes most to seed production in the Norwegian populations (Sletvold *et al.*, 2012), whereas nocturnal pollination contributes most in the Swedish populations (Chapurlat *et al.*, 2015; E. Chapurlat, unpublished data). These populations are visited by pollinators belonging to three functional groups: in all populations, nocturnal Lepidoptera dominate at night and diurnal Lepidoptera dominate during the day, except in one of the Norwegian populations, where *Empis* flies are the dominating daytime visitors (Sletvold *et al.*, 2012). This variation in the relative importance of diurnal and nocturnal pollinators and in dominating functional groups that are likely to have different physiological responses to floral scent compounds should result in selection for different scent composition and emission rhythm between the day and night. Here, we quantified floral scent emission rates and scent composition during the day and night in the field in the six populations. Moreover, we tested whether the patterns detected in the field persisted when plants from two populations in each region were transferred to a common environment.

If floral scent emission patterns in *G. conopsea* have evolved in response to local pollination regimes, we predicted that (1) scent emissions should be highest at night in the Swedish populations and during day in the Norwegian populations; (2) scent composition should differ between day and night; and (3) the Norwegian population dominated by *Empis* should differ from the other populations in diurnal scent composition.

MATERIALS AND METHODS

Study species and populations

Gymnadenia conopsea is a terrestrial orchid distributed across Eurasia (Hultén and Fries, 1986). It occurs on calcareous soils in grazed meadows, and in margins of marshes and fens. The species is a tuberous, non-clonal and long-lived perennial (Øien and Moen, 2002). The fragrant flowers vary in colour from pale to bright pink, and rarely white. Flowers open sequentially from the bottom to the top of a single inflorescence of 10–100 flowers. Individual flowers remain open for up to a week, while individual plants may flower for a month. A long, narrow spur contains nectar that is produced throughout anthesis (Stpiczynska and Matusiewicz, 2001). Each flower contains two pollinaria, which are situated above the spur entrance. Plants are self-compatible, but depend on pollinators for successful fruit set (Sletvold *et al.*, 2012).

We studied a total of six populations, four in southern Sweden and two in central Norway, for which we have information on the common pollinators and periods of visit peaks during the day and night (Table 1). Table 1 lists all visitors observed in the study populations based on a combination of observations and video recordings during the day and at night. In five of the populations, the relative importance of diurnal and nocturnal pollinators for seed production has been established experimentally (Sletvold *et al.*, 2012; Chapurlat *et al.*, 2015; E. Chapurlat unpubl. res.). Nocturnal and diurnal pollinator visitation rates, based on video camera recordings in four of the six populations, show a pattern consistent with the selective pollinator exclusion experiments. In two of the Öland populations, recordings during peak visitation

TABLE 1. *Study population characteristics and pollinators observed on* Gymnadenia conopsea *(T, temperature during scent collection)*

Country	Population name and location	Population size (flowering individuals)	Peaks of diurnal (D) and nocturnal (N) visits and mean T	Diurnal visitors	Nocturnal visitors	Pattern of relative abundance of diurnal (D) and nocturnal (N) pollinators
Sweden	Folkeslunda 56°43′N 16°44′E	Approx. 2000	D: 10.00–14.00 h, 21.3 °C N: 22.00 –24.00 h, 12.7 °C	*Aglais urticae*	*Autographa gamma* *Cucullia umbratica* *Deiliphila porcellus*	D < N
Sweden	Kvinneby 56°33′N 16°37′E	400–600	D: 10.00–14.00 h, 24.2 °C N: 22.00 –24.00 h, 13.4 °C	*Aglais urticae* *Pieris brassicae*	*Autographa gamma* *Deiliphila porcellus* *Hyles gallii*	D < N
Sweden	Långlöt 56°45′N 16°45′E	Approx. 1000	D: 10.00–14.00 h, 20.7 °C N: 22.00 –24.00 h 13.2 °C	*Aglais urticae* *Empis* sp. *Siona lineata*	*Autographa gamma* *Cucullia umbratica* *Deiliphila porcellus* *Hyles gallii*	D < N
Sweden	Melösa 56°51′N 16°50′E	~2500	D: 10.00–14.00 h, 20.7 °C N: 22.00–24.00 h, 7.9 °C	*Aglais urticae* *Zygaena minos*	*Agrotis exclamationis* *Autographa gamma* *Deiliphila porcellus*	D < N
Norway	Sølendet 62°40′N 11°50′E	500–1000	D: 10.00–14.00 h, 15.3 °C N: 23.00–02.00 h, 7.4 °C	*Boloria* sp. *Empis tessellate* *Pyrgus centaurea*	*Hyles gallii* *Papestra biren*	D > N
Norway	Tågdalen 63°03′N 09°05′E	200–600	D: 10.00–14.00 h, 16.7 °C N: 23.00–02.00 h, 6.1 °C	*Aglais urticae* *Boloria* sp. *Hemaris tityus*	*Autographa pulchrina* *Hyles gallii*	D > N

Pollinator data compiled from Sletvold *et al.* (2012), Chapurlat *et al.* (2015) and unpublished video recordings.

In all populations except Kvinneby, the relative importance of diurnal and nocturnal pollinators for seed production has been established experimentally.

during the night and day (16–20 h per period and population) documented high nocturnal visitation rates from the equally common *Deilephila porcellus* and *Autographa gamma* (in total 7.2 and 6.1 visits h^{-1} in Kvinneby and Folkeslunda, respectively), but not a single visit from diurnal pollinators. In contrast, no visits were documented in the Norwegian Sølendet population during 25 h of nocturnal recording, whereas *Empis* flies visited frequently during the day (N. Sletvold, pers. obs., no video recording). In the Norwegian Tågdalen population, both nocturnal and diurnal visitation was documented during 6 h of recording in each period, with *Autographa pulchrina* being the only nocturnal visitor (0.83 visit h^{-1}) and *Hemaris tityus* the only diurnal visitor (2.1 visits h^{-1}).

Conditions for scent sampling in the field

To quantify floral scent variation in the field, we sampled the six natural populations using dynamic headspace adsorption in 2013. At peak flowering, we individually marked and sampled between 14 and 22 individuals per population. For each individual, volatiles were sampled for 1 h during both the day and night. In each population, the timing of sampling was matched to the peak pollinator activity (Table 1): day sampling started between 12.00 h and 15.00 h, and night sampling started between 22.00 h and 23.00 h in the Swedish populations and between 23.00 h and 01.00 h in the Norwegian populations. We also recorded air temperature at the beginning and end of each sampling occasion (averages given in Table 1). In each population, day and night sampling were conducted over two consecutive days.

Conditions for scent sampling in growth chambers

To quantify scent variation in a common environment, we moved plants from the two Norwegian and two Swedish populations to growth facilities at Uppsala University, Uppsala, Sweden. In summer 2015, we dug up 20 bolting plants in each of the Melösa, Långlöt and Sølendet populations, and ten plants in the smaller Tågdalen population. Plants were put in 10 × 10 cm pots filled with surrounding soil and brought within 2 d to the Uppsala University facilities. The plants were placed in growth chambers (BioChambers-SPC-56, Winnipeg. Manitoba) with temperature and photoperiod settings intermediate to field conditions in Norway and Sweden (see Table 1): 15 h day, light intensity 800 µE, 20 °C; 2 h dusk, 80 µE, 15 °C; 5 h night, 0 µE, 10 °C; 2 h dawn, 80 µE, 15 °C. Once plants were in full bloom, after 13–20 d of growing in pots, two samples of floral scent were taken from each plant: night sampling was performed between 1 and 3 h after dusk, and day sampling was performed between 8 and 10 h after dawn, to mimic sampling time in the field. As in the field, sampling equipment at night was installed in the light of a headlamp.

Dynamic headspace scent sampling and scent sample preparation

For each individual, we counted the number of open flowers at the time of sampling. To control for a potential damage effect of sampling, half of the plants in each population were sampled first during the day, while the other half were sampled first at night. No damage effect was identified (no apparent wounding compounds were detected during the second collection bout, and there was no clustering of samples based on sampling order in non-metric multidimensional scaling (NMDS) plots used for graphical exploration of the multivariate data, see 'Statistical analyses' below). Inflorescences were enclosed in Toppits® (for the field samples) or ICA® (for the growth chamber samples) oven bags together with a Teflon tube scent trap filled with 10 mg of a Tenax GR® filter. Air was extracted from the bags through a small hole at the top of the bag by a battery-operated

vacuum pump (GroTech, Gothenburg, Sweden) maintaining a steady flow of 200 mL min^{-1}. The air flow was continuously monitored by a Cole-Parmer (Vernon Hills, IL, USA) 65 mm direct-reading flow meter. Volatiles were sampled for 1 h. Before use, traps were cleaned with acetone and hexane. At each sampling occasion, a control sample of ambient air was collected to identify background contamination. After sampling, adsorbed volatiles were eluted from the traps with 300 µL of gas chromatography/mass spectrometry (GC/MS) quality hexane (Suprasolv®; VWR). Samples were sealed in glass vials and stored at –20 °C before being concentrated to 50 µL under a constant moderate flow of nitrogen gas. An internal standard of 5 µL of 0.03 % toluene solution (1.3 µg) in hexane was added to each sample to allow an estimation of the standardized emission rate for quantitative comparisons using the method from Svensson *et al.*, (2005). Samples were stored at –20 °C until analysis. The total emission rate of compounds from an inflorescence was calculated as follows:

$$\textit{Standardized emission rate per inflorescence}\ (\mu g) = \frac{\sum_i \frac{\textit{peak area of compound } i}{\textit{peak area of internal standard}}}{\textit{hours of sampling}} \times \textit{amount of internal standard added}\ (\mu g)$$

The standardized emission rate per flower was calculated by dividing the standardized emission rate per inflorescence by the number of open flowers at the time of sampling.

Quantitative GC/MS analysis

Scent samples were analysed using a Finnigan TraceGC ultra 2000 gas chromatograph connected to a Finnigan TraceDSQ mass spectrometer (both Thermo Fisher, USA). The gas chromatograph was equipped with a 30 m × 0.250 mm × 0.25 µm DB-Wax column (Agilent Technologies, USA). Helium was used as carrier gas at a constant velocity of 1 mL min^{-1}. The temperature program started with a 3 min hold at 50 °C, after which the oven temperature increased by 10 °C min^{-1} for 20 min until it reached a maximum temperature of 250 °C. The program ended with a 7 min hold at this temperature. The floral volatile peaks in the chromatograms were manually integrated using the mass spectrometer manufacturer's software (Xcalibur™ version 1.4, ®Thermo Electron Corporation 1998–2003). Most compounds were identified by verification of MS library suggestion (NIST version 2.0, 2008) using Kovats retention index values obtained from the literature for wax columns equivalent to the DB-Wax column used in this study, and some using authentic standards rerun on the GC/MS system (the identification method and Kovats index references are given in Supplementary Data Table S1). The remaining compounds were denoted as unknowns (the relative abundance of the ten most abundant ions of these compounds is provided in Table S1).

Statistical analyses

All analyses were conducted in R 3.1.3 (R Core Team, 2015), except for the planned contrasts that were run in SAS 9.03 (2002–2010), executing on the X64 7Pro platform (SAS Institute, Cary, NC, USA and Microsoft, Redmond, WA, USA).

To test whether total floral scent emission rates varied among populations and differed between day and night, we analysed the standardized emission rate per inflorescence and per flower with a repeated measures analysis of variance (ANOVA) including sampling period (day or night) as a within-subject factor, population as a between-subject factor, and their interaction. In this analysis, a significant interaction term indicates that the difference between diurnal and nocturnal emission varies among populations. We used type-III SS tests by using the 'ezANOVA' function of the 'ez' package (Lawrence, 2015). We used planned contrasts to test if the effect of period (day vs. night) on scent emission differed between the two Norwegian populations mainly pollinated by diurnal pollinators and the four Swedish populations mainly pollinated by nocturnal pollinators (Contrast period). Pairwise *t*-tests were used to determine in which populations the difference between day and night was statistically significant. We analysed field and growth chamber data in separate analyses. Standardized emission rates were log transformed prior to analyses to meet assumptions of normality and homoscedasticity of residuals.

To examine qualitative variation in the floral scent bouquets, we conducted multivariate analyses of the untransformed proportional contribution of each compound with the 'vegan' package (Oksanen *et al.*, 2015). Differences in scent composition between groups of samples (populations and period of sampling) were first explored graphically, by plotting the pie charts representing the average scent composition for each period in each population both in the field and in the growth chamber (Fig. 1), and through the graphical output of NMDS with different symbols for each group (Supplementary Data Fig. S1). We also examined variation among periods and populations with non-parametric multivariate analysis of variance (perMANOVA, function 'adonis', 10 000 permutations). The output of the perMANOVA provides an estimate of how much variation in the data is explained by the factor of interest (represented by its R^2). We chose not to interpret *P*-values, as significance testing in this analysis is sensitive to heterogeneous dispersion when data are unbalanced (Anderson and Walsh, 2013). We analysed field and growth chamber data in separate analyses. Because the most important group distinction was a difference between diurnal and nocturnal samples, we conducted a SIMPER analysis ('simper' function) on proportions to determine which compounds contributed the most to the dissimilarity between diurnal and nocturnal samples. Finally, we tested whether absolute emission rates of each compound increased or decreased at night, based on all field scent samples. Because the data were not normally distributed and each plant was sampled both duting the day and at night, we conducted paired Wilcoxon tests comparing absolute rates during the day and at night for each compound.

RESULTS

In total, we detected 66 floral compounds in the *Gymnadenia conopsea* scent samples, out of which one was an unidentified sesquiterpene and one was completely unknown (Supplementary Data Table S1). The unknown compounds represented very low percentages in the scent samples, varying, when present, from an average of <0.001 % to 0.28 % among period × population × sampling condition combinations (Table

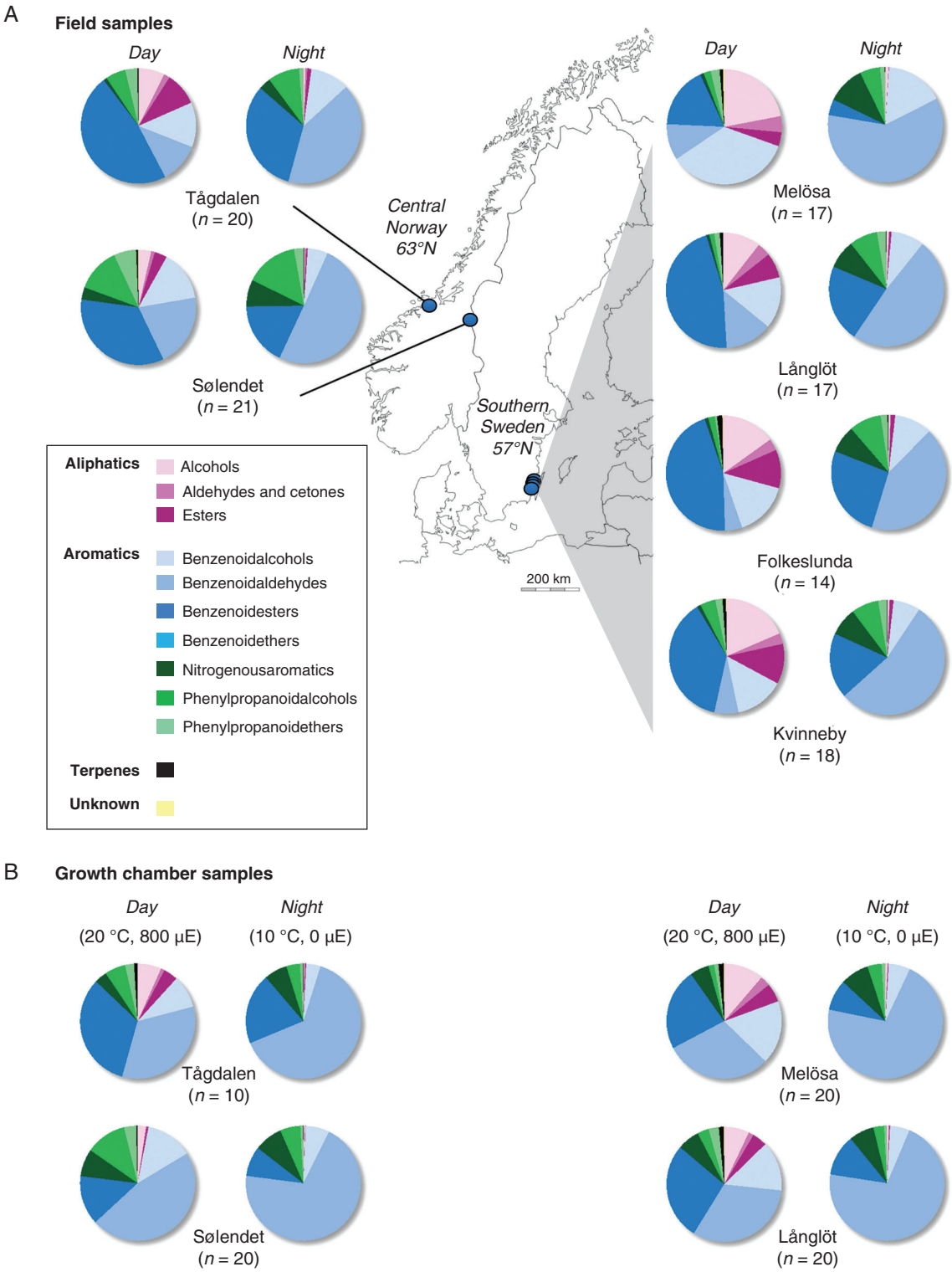

FIG. 1. Floral scent variation in the diurnal (Day) and nocturnal samples (Night) presented as pie charts showing the relative contribution of different compound groups to the floral scent bouquet in the two types of samples for each of the six *Gymnadenia conopsea* populations sampled: (A) in the field in 2013 and (B) in the growth chamber in 2015.

S1). The floral bouquet was largely dominated by aromatic compounds, but also included aliphatics and small proportions of mono- and sesquiterpenoids (Fig. 1; Table S1). Individual floral scent samples included between five and 50 compounds (median 23 compounds), and the range was similar for all populations. The number of compounds detected tended to increase with total emission rate (correlation with log-transformed scent emission rate, $R = 0.41$, $n = 354$).

Variation in total floral scent emission rates among periods, populations and regions

In both the field and growth chamber environments, the effect of period on per-inflorescence scent emission rate varied among populations (Table 2; significant population × period interactions). In the field, the difference between nocturnal and diurnal emission was greater in the four Swedish populations than in the two Norwegian populations (Table 2: significant × period contrast, Fig. 2A). The larger difference was caused by higher nocturnal emission in the Swedish populations, whereas diurnal emission did not significantly differ between the two categories of populations (Table 2; Fig. 2A). As predicted, emission was higher at night than during the day in Sweden (statistically significant in three of four populations, paired *t*-test, $P < 0.05$, Fig. 2A), but the opposite was not the case in Norway. Although the effect of period was smaller in Norway, both populations emitted more scent at night than during the day ($P < 0.05$, Fig. 2A). Also in the growth chamber, the difference between nocturnal and diurnal emission was greater in the two Swedish populations compared with the two Norwegian populations (Table 2: significant population × period contrast, Fig. 2B). Nocturnal scent emission rates were significantly higher in Swedish populations compared with Norwegian populations, whereas the opposite was true for diurnal emission rates (Table 2). The latter was due to a markedly higher diurnal scent emission rate in the Sølendet population (Fig. 2B). Scent emission was significantly higher at night than during the day in Sweden and in the Norwegian Tågdalen population, whereas the pattern was reversed in the Norwegian Sølendet population (paired *t*-tests, all $P < 0.05$, Fig. 2B). In the growth chamber, all four populations emitted more scent than in the field, during both the day and night (Fig. 2).

The results were overall similar when scent emission rate per flower was analysed, both in the field and in the growth chamber. The largest difference was seen in the Sølendet population sampled in the field. Here, plants exhibited higher per-flower emission rates than in any of the other populations both during the day and at night (Supplementary Data Fig. S2; Table S2), due to high scent emission rates per inflorescence despite having few open flowers at the time of sampling (Fig. 2; Supplementary Data Fig. S2).

In the field, day and night emissions per inflorescence were positively correlated in all populations (log-transformed, Pearson $r = 0.42–0.61$, $P < 0.05$) except Sølendet ($r = 0.13$, $P = 0.59$). In contrast, day and night emissions were not correlated in the growth chamber. Finally, there was a significant positive correlation between temperature during scent sampling and emission rate in the field at night but not during the day (Supplementary Data Fig. S3).

TABLE 2. *The effects of population, sampling period (day vs. night) and their interaction on the standardized emission rate (SEM) of floral scent per inflorescence of* Gymnadenia conopsea *in the field and in the growth chamber analysed in a repeated measures ANOVA, and planned contrasts for the comparison of day and night emissions between the two sets of populations (Sweden vs. Norway)*

SEM per inflorescence (log-transformed)	Field ($n = 107$)		Growth chamber ($n = 70$)	
	F	*P*-value	*F*	*P*-value
Population	20.00	**<0.001**	8.063	**<0.001**
Period	109.5	**<0.001**	35.11	**<0.001**
Population × period	3.872	**0.003**	12.35	**<0.001**
Contrast day	0.64	0.42	8.68	**0.0044**
Contrast night	5.35	**0.023**	6.30	**0.0145**
Contrast period	8.42	**0.0046**	16.39	**<0.001**

Significant effects are in bold.

Variation in floral scent composition

In both the field and the growth chamber, floral scent composition differed markedly between day and night, but was rather consistent across populations and regions (Fig. 1). The period of sampling explained the largest part of the variation in scent composition among both field samples (perMANOVA: $R^2 = 0.34$, Supplementary Data Fig. S1A) and growth chamber samples (perMANOVA: $R^2 = 0.35$, Supplementary Data Fig. S1B), while among-population variation explained considerably less variation (perMANOVA: $R^2 = 0.08$ in the field, Supplementary Data Fig. S1C; and $R^2 = 0.09$ in the growth chamber, Fig. S1D). During the day, in Sølendet, aliphatics represented a lower proportion while benzenoid aldehydes, nitrogenous aromatics and phenylpropanoids were emitted in greater proportion compared with other populations (Fig. 1), but these differences did not lead to a distinct grouping of these samples in the NMDS plots (Supplementary Data Fig. S1). The nine most abundant compounds accounted for 80 % of the day vs. night dissimilarity (SIMPER analysis, non-transformed proportion data, Table 3). Phenylacetaldehyde alone explained 29.7 % of the dissimilarity, representing a greater proportion of the scent bouquet at night than during the day (Table 3), due to a 10-fold increase in absolute emission rate at night (Fig. 3). 2-Phenethyl acetate and 2-phenylethanol represented lower proportions during the night than during the day (Table 3), but this was caused by the nocturnal increase in the proportion of phenylacetaldehyde rather than a decrease in their absolute emission rates, which also increased significantly at night (Fig. 3). Of the 65 compounds detected in field samples, 52 exhibited significant differences in absolute emission rates between day and night (Fig. 3). The emission rate of 21 of the 31 aliphatics increased significantly during the day, while only two showed the opposite pattern (Fig. 3A). Eight of nine terpenes and the unknown compound also showed a significant increase of their emission rates during the day (Fig. 3C). In contrast, the absolute emission rates of the majority of aromatic compounds (15 out of 24 compounds) increased at night, while only five showed the opposite pattern (Fig. 3B).

DISCUSSION

This study compared floral scent production of four Swedish *Gymnadenia conopsea* populations pollinated predominantly at night with that of two Norwegian populations pollinated predominantly during the day. In line with predictions, scent composition differed markedly between day and night, and the emission rate increased at night in the Swedish

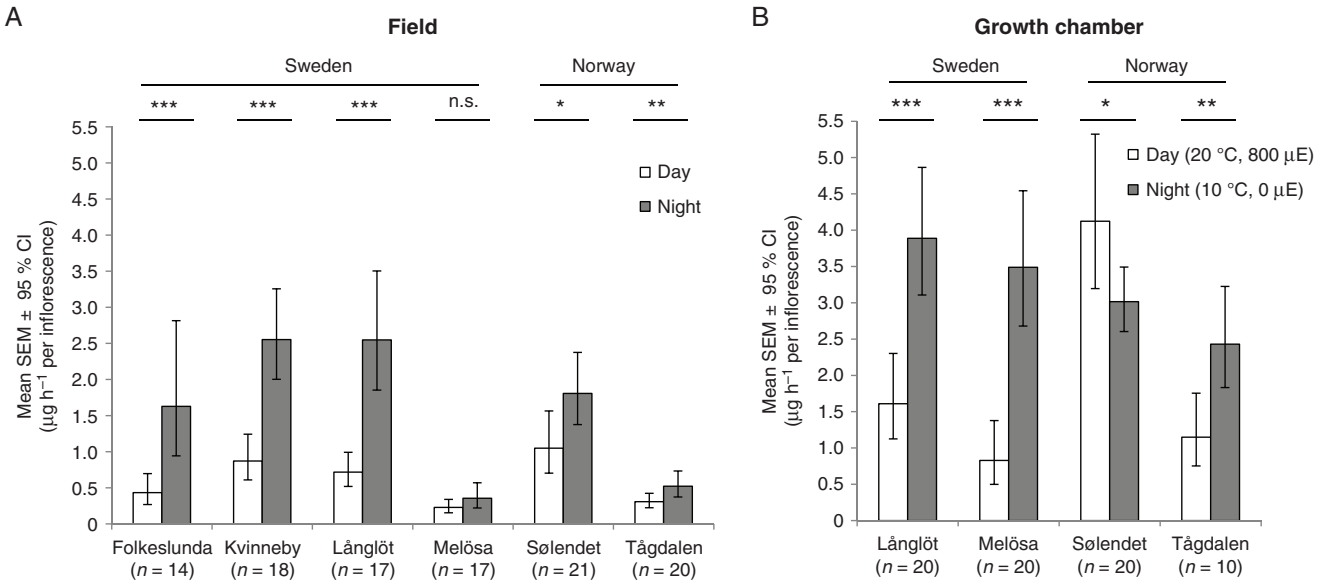

FIG. 2. Mean floral volatile standardized emission rate (SEM) per inflorescence ± 95 % confidence interval (back-transformed from the log-transformed data) in the field (A) or in the growth chamber (B) and in diurnal and nocturnal conditions for the two sets of *Gymnadenia conopsea* study populations located in Sweden and Norway. The statistical significance of the difference between diurnal and nocturnal SEM within each population (tested with a paired *t*-test on log-transformed data) is indicated. n.s., not significant $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

populations. However, contrary to prediction, the emission rate also increased at night in the Norwegian populations, although the difference between day and night was significantly smaller. Among-population variation in scent composition was modest, but certain compounds represented higher proportions in Norwegian than in Swedish populations, and particularly so in the Sølendet population where *Empis* flies are important diurnal pollinators. The results suggest that the relative importance of nocturnal and diurnal pollination influences the evolution of floral scent emission rhythms.

The rate, rhythm and composition of floral scent emissions all are known to show plasticity in response to environmental factors (Hansted *et al.*, 1994; Jakobsen and Olsen 1994; Gouinguene, 2002; Farré-Armengol *et al.*, 2014; Friberg *et al.*, 2014; Majetic *et al.*, 2017), which makes it important to complement field observations of scent variation with data from a controlled environment. We found that diel patterns of scent emission largely persisted in a common environment, but that the relative emission rates of different populations changed, suggesting that patterns in the field were a combination of plasticity and genetically based differences. In our field study, one Swedish population, Melösa, and one Norwegian population, Tågdalen, emitted considerably less scent than the other populations both during the day and at night, but this difference did not persist when plants were transferred to the growth chamber (Fig. 2). Given the average temperatures during sampling in these populations (Table 1) and the positive correlation between temperature and the night-time scent emission rate (Supplementary Data Fig. S3), variation in ambient temperature can explain their lower nocturnal emission rates but not their lower diurnal emission rates. Instead, other factors, such as differences in air or soil humidity, may have contributed to the variation observed (Gouinguene, 2002). The difference in emission rate between day and night was larger in the Swedish populations compared with the Norwegian populations also after transfer to the growth chamber (Fig. 2). Because the plants were moved at the bolting stage, conditions during the pre-bolting stages might have influenced scent emission rates during later flowering, but this is unlikely as floral scent rhythms have been shown to respond within hours to changes in temperature or photoperiod (Altenburger and Matile, 1988; Hansted *et al.*, 1994; Jakobsen and Olsen, 1994). Our results thus suggest a genetic component to variation in diel scent rhythms among *G. conopsea* populations.

The observed among-population differences in diel rhythms are partly consistent with the prediction that the timing of floral scent emissions should match the peak period of pollinator activity. Scent emissions were greater at night than during the day in the four Swedish populations, but the opposite was not

TABLE 3. *Compounds contributing to the first 80 % of average Bray–Curtis dissimilarity based on untransformed proportion data between headspace scent samples collected from* Gymnadenia conopsea *in the field during different periods (D, day; N, night) obtained from SIMPER analysis.*

Compound	Percentage of D vs. N dissimilarity explained (all *P*-values <0.001)	Pattern
Phenylacteladehyde	29.7 %	N > D
2-Phenylethyl acetate	21.3 %	D > N
2-Phenylethanol	12.8 %	D > N
1-Tetradecanol	4.4 %	D > N
Indole	3.9 %	N > D
Eugenol	2.9 %	N > D
1-Hexadecanol	2.7 %	D > N
E-Isoeugenol	1.9 %	N > D
Z-7-Dodecenyl acetate	1.6 %	D > N
All nine compounds	81.2 %	–

Overall between-group dissimilarity = 0.642.

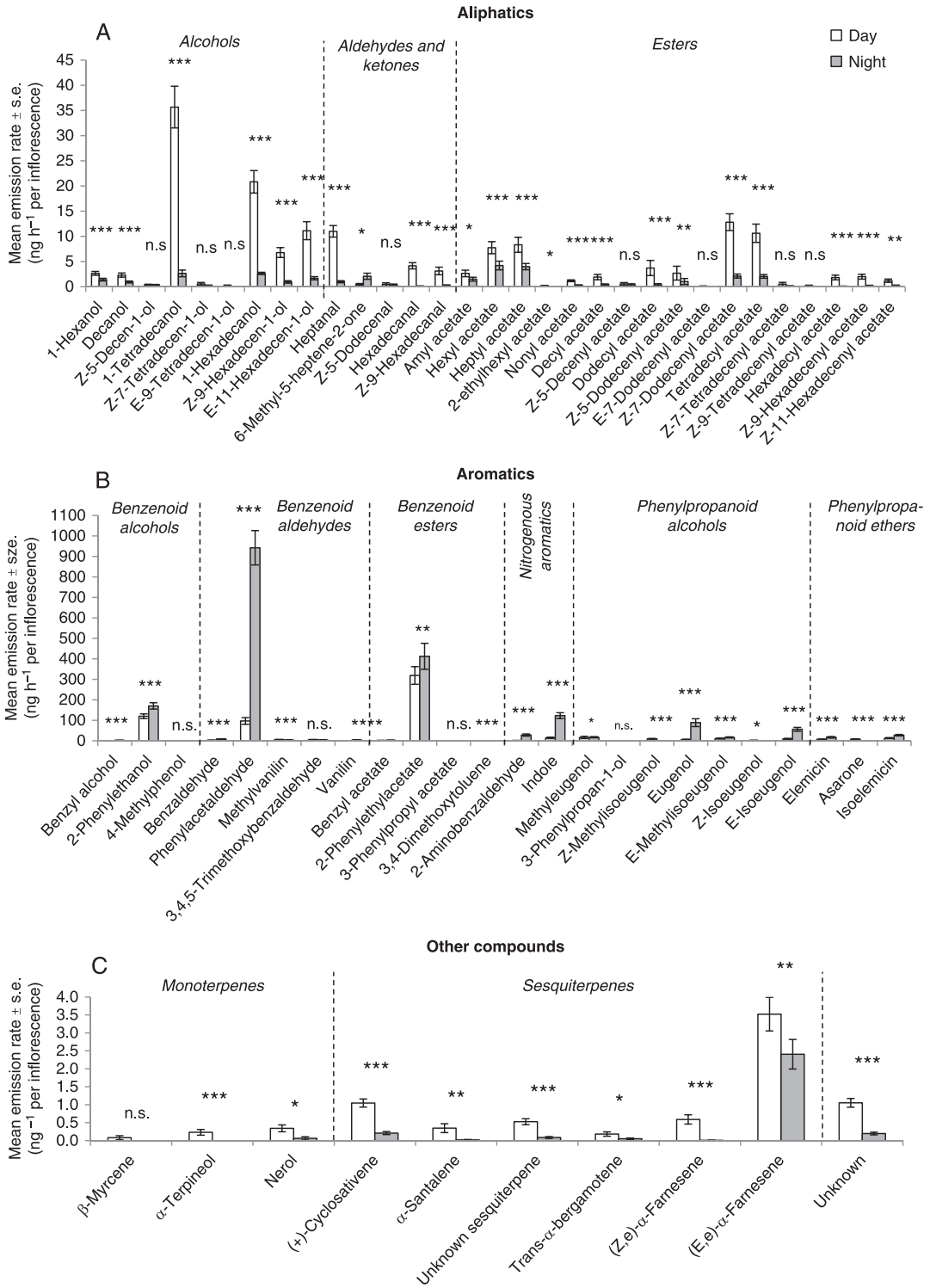

FIG. 3. Mean absolute emission rates per inflorescence ± s.e. during the day and night for all 65 floral scent compounds detected in the floral bouquet of the orchid *Gymnadenia conopsea* in the field in 2013 (mean based on $n = 107$ plants sampled in six populations). Compounds are grouped by chemical type: aliphatics (A), aromatics (B), and terpenes and other compounds (C). For each compound, the statistical significance of the difference in emission rates between day and night tested with a paired Wilcoxon test is indicated. n.s., not significant $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

true in the two Norwegian populations. This could be because floral scent is a more important signal at night than during the day, while visual display may be more important for attracting day-active pollinators. Studies on several lepidopteran species indicate that the relative importance of visual and olfactory signals at night may be interaction-specific. Scent is clearly the most important cue in some systems (Balkenius *et al.*, 2005), whereas a combination of visual and olfactory cues is necessary

in others (Raguso and Willis, 2005; Hirota *et al.*, 2012). Still, the observation that plants from the Sølendet population emit significantly more diurnal scent compared with the other populations suggests that floral scent could be important for attracting the diurnal *Empis* flies that visit this population.

Plants pollinated during both the day and night encounter two guilds of pollinators belonging to different functional groups (*sensu* Fenster *et al.*, 2004), and it thus can be expected that the composition of the floral scent bouquet should differ between day and night. Indeed, all studies that have characterized floral scent in such study systems, including the current study, recorded a change in the composition of the floral scent bouquet between day and night (Huber *et al.*, 2004; Morinaga *et al.*, 2008; Dötterl *et al.*, 2012; Jürgens *et al.*, 2014; van der Niet *et al.*, 2015; Prieto-Benítez *et al.*, 2016). In one case, this variation could be linked to the preferences of diurnal and nocturnal pollinators shown in bioassays (Jürgens *et al.*, 2014). In the present study, most of the compounds in *G. conopsea* floral scent exhibited significant variation in absolute emission rates between day and night. In general, the emission of aromatics increased at night, most dramatically that of phenylacetaldehyde, whereas the emission of aliphatics and terpenes decreased at night. Phenylacetaldehyde emissions have been reported to increase at night also in two closely related *Gymnadenia* congeners, which likewise are pollinated by lepidopteran pollinators during both the day and night (Huber *et al.*, 2004). In other systems, there are examples where aromatics or terpenes showed diel patterns similar to this study (Morinaga *et al.*, 2008; Dötterl *et al.*, 2012) or the opposite patterns, with aromatics being more dominating during daytime (Friberg *et al.*, 2014). Interestingly, in these few examples, an increase of aromatics seems generally to occur during the period when Lepidoptera contribute the most to pollination, whether it is during the day (Friberg *et al.*, 2014) or at night (Dötterl *et al.*, 2012; this study). This further supports the notion that peak scent emission is tied to pollinator activity rather than abiotic factors alone.

We expected that scent composition of diurnal samples in the Sølendet population should differ the most from that of the other populations because the major diurnal pollinators, the empidid flies, belong to a different functional group (*sensu* Fenster *et al.*, 2004). Most compounds were shared by all populations, and variation in the relative proportions of the compounds was limited. Still, the Sølendet population differed the most from the other populations (Fig. 1). The presence of empidid flies in this population may have selected for a different floral scent composition, as hypothesized in a study of the sister species *G. odoratissima* to explain the differences in floral scent between lowland and mountain populations (Sun *et al.*, 2014). In their study of *G. odoratissima*, Sun and colleagues found that plants from populations where empidid flies are present emitted fewer aromatics but more aliphatics than plants from other populations. In contrast, the proportion of aromatic compounds was either similar or even higher (for phenylacetaldehyde, eugenol and methyleugenol, Supplementary Data Table S1) in the *Empis*-dominated Sølendet population compared with other populations in our study, and vice versa for the aliphatic compounds (Table S1). This indicates that if selection by empidid flies contributes to local scent variation, it has targeted different floral compounds in the different systems.

Other differences in scent composition among our populations were limited, especially at night. The pollinator communities among our populations varied mainly in species belonging to the same two functional groups, i.e. diurnal and nocturnal lepidopterans, with some species shared by most populations, such as *Aglais urticae* or *Hyles gallii* (Table 1). It is possible that the floral scent of *G. conopsea* is composed of compounds that attract many different pollinator species. Phenylacetaldehyde, 2-phenylethyl acetate and 2-phenylethanol, the three dominant compounds in our floral scent samples, have been reported in the floral scent of many plant species (Knudsen *et al.*, 1993; El-Sayed, 2016) and are known to elicit antennal responses and attract various insect species, in particular Lepidoptera (Plepys *et al.*, 2002; Huber *et al.*, 2004; Dötterl *et al.*, 2006; Svensson *et al.*, 2010, and references therein) and Diptera (Zhu *et al.*, 2003; Zhu and Park, 2005; Jhumur *et al.*, 2007; Stökl *et al.*, 2010, and references therein). *Gymnadenia conopsea* might have evolved a floral scent bouquet that differs between day and night to better attract, respectively, diurnal and nocturnal pollinators, but that can attract a wide range of species within each category.

In a few systems, floral scent has been shown to mediate interactions with antagonists (e.g. Theis, 2006; Andrews *et al.*, 2007), and among-population variation in diel scent rhythms could thus also be influenced by spatial variation in antagonist-mediated selection. However, we have no indication of relevant variation in interactions with antagonists among our study populations. Neutral processes and history could also have contributed to divergence in floral scent. The four Swedish populations with similar diel scent rhythms are closely located and likely to be well connected by gene flow, while the two Norwegian populations, which differ from each other and from the southern populations in their scent emission rhythms, are perhaps more isolated, less connected by gene flow and thus potentially more sensitive to genetic drift. To disentangle further the roles of adaptive and non-adaptive processes for diel scent variation, experimental studies are needed to identify the targets and agents of selection on floral scent.

Conclusion

This is the first study to report among-population variation in floral scent emission rhythms in the field supported by measurements in controlled conditions. Previous studies of spatial variation in floral scent have largely focused on scent composition, but this study indicates that both aspects should be considered for a full understanding of variation in floral scent phenotypes. Our results are consistent with a scenario in which the diel variation in scent variation has been driven predominantly by plant–pollinator interactions. An important next step is to quantify selection on scent composition and emission rates mediated by diurnal and nocturnal pollinators in this and other systems.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: NMDS plots of the *G. conopsea* scent samples showing the influence of period (day vs. night) on the composition of field samples

and growth chamber samples, and the among-population differences in scent composition of field samples and growth chamber samples. Figure S2: mean floral volatile standardized emission rate per flower in the field and in the growth chamber and in diurnal and nocturnal conditions for the two sets of *G. conopsea* study populations located in Sweden and Norway. Figure S3: correlation between mean standardized emission rate per inflorescence and temperature during the scent sampling in the field during the day and at night. Table S1: list of floral scent compounds in Excel with Kovats index, identification method, literature references and mean proportion in the scent bouquet of *G. conopsea* during the day and at night in each study population, in the field and in the growth chamber. Table S2: the effects of population, sampling period and their interaction on the standardized emission rates of floral scent per flower of *Gymnadenia conopsea* plants in the field and in the growth chamber analysed in a repeated measures ANOVA, and planned contrasts for the comparison of the two sets of populations (Sweden vs. Norway).

ACKNOWLEDGEMENTS

We thank Amélie Truchy for her valued help in the field, Amy Parachnowitsch and Sophie Karrenberg for their help with the design of the growth chamber experiment, Glenn Svensson from Lund University for providing some standard compounds, and two anonymous reviewers for helpful comments on an earlier version of the manuscript. This work was supported by grants from Extensus Stiftelsen and Linnéska stipendiestiftelsen to E.C., from Letterstedska Stiftelsen to M.F. and N.S., from Längmanska Kulturfonden and Helge Ax:son Johnsons Stiftelse to M.F., from The Swedish Research Council to J.Å. and M.F., and from The Swedish Research Council Formas to N.S.

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