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PART OF A SPECIAL ISSUE ON POLLINATOR-DRIVEN SPECIATION

Floral adaptation to local pollinator guilds in a terrestrial orchid

Mimi Sun*, Karin Gross and Florian P. Schiestl

Institute of Systematic Botany, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland
** For correspondence. E-mail mimi.sun@systbot.uzh.ch*

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• *Background and Aims* Studies of local floral adaptation in response to geographically divergent pollinators are essential for understanding floral evolution. This study investigated local pollinator adaptation and variation in floral traits in the rewarding orchid *Gymnadenia odoratissima*, which spans a large altitudinal gradient and thus may depend on different pollinator guilds along this gradient.
• *Methods* Pollinator communities were assessed and reciprocal transfer experiments were performed between lowland and mountain populations. Differences in floral traits were characterized by measuring floral morphology traits, scent composition, colour and nectar sugar content in lowland and mountain populations.
• *Key Results* The composition of pollinator communities differed considerably between lowland and mountain populations; flies were only found as pollinators in mountain populations. The reciprocal transfer experiments showed that when lowland plants were transferred to mountain habitats, their reproductive success did not change significantly. However, when mountain plants were moved to the lowlands, their reproductive success decreased significantly. Transfers between populations of the same altitude did not lead to significant changes in reproductive success, disproving the potential for population-specific adaptations. Flower size of lowland plants was greater than for mountain flowers. Lowland plants also had significantly higher relative amounts of aromatic floral volatiles, while the mountain plants had higher relative amounts of other floral volatiles. The floral colour of mountain flowers was significantly lighter compared with the lowland flowers.
• *Conclusions* Local pollinator adaptation through pollinator attraction was shown in the mountain populations, possibly due to adaptation to pollinating flies. The mountain plants were also observed to receive pollination from a greater diversity of pollinators than the lowland plants. The different floral phenotypes of the altitudinal regions are likely to be the consequence of adaptations to local pollinator guilds.

Key words: Local adaptation, pollination, floral evolution, geographical variation, floral morphology, floral scent, VOC, floral colour, pollinator assemblages, pollinator adaptation, Diptera, Orchidaceae, speciation.

INTRODUCTION

The adaptation of plants to different pollinators is widely regarded as a key mechanism promoting the diversification and speciation of animal-pollinated angiosperms (Grant and Grant, 1965; Stebbins, 1970; Faegri and van der Pijl, 1979; Schluter, 2000; Johnson, 2006; Schiestl and Schlüter, 2009; Schiestl, 2012). Several lines of evidence support the link between animal pollinators and angiosperm diversification. These include the sudden and broad diversification of animal-pollinated plant lineages (Eriksson and Bremer, 1992; Ricklefs and Renner, 1994; Dodd *et al.*, 1999), strong selection exerted on floral traits by pollinators (e.g. Galen, 1989; Campbell *et al.*, 1997; Schiestl and Johnson, 2013) and floral phenotype associations with particular pollinator groups (Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003; Fenster *et al.*, 2004; Willmer, 2011; Schiestl and Dötterl, 2012).

The first conceptual model of pollinator-driven speciation was developed by Grant and Grant (1965), who noted in a study of *Gilia leptantha* (Polemoniaceae) that the floral trait variation across a geographical range appeared to have derived from a pollinator-shift between bees and bee-flies. Stebbins (1970) expanded on the concept that divergence in floral form is often attributed to variation in geographical pollinator mosaics and adaptation to the most effective pollinator. As different pollinators vary in functional morphology, foraging behaviour, thermal biology, nutritional requirements and innate floral preferences, the geographical variability in pollinator composition could result in divergent selection pressures on floral traits between intraspecific populations. Selection mosaics on floral traits that enhance reproductive success will induce the evolution of locally adapted variants of a species. Ultimately, if the different pollination 'ecotypes' were to arrive into secondary contact, the pollinator preferences could conceivably prevent any intercrossing.

Although the Grant-Stebbins model (Johnson, 2006) is the basis for allopatric and parapatric divergence in pollination systems, studies of the role of geographical variation in pollinators and intraspecific floral adaptation to local pollinators in floral diversification remain relatively sparse (as reviewed by Coyne and Orr, 2004; Herrera *et al.*, 2006; Johnson, 2006). Recent approaches to research on the adaptive origin of floral diversity have focused on the correlation between floral diversification and pollination in the light of phylogenetics (e.g. Hapeman and Inoue, 1997; Graham and Barrett, 2004; Patterson and Givnish, 2004; van der Niet and Johnson, 2012), or on pollinator-mediated phenotypic selection on floral traits within a single natural population (e.g. Campbell *et al.*, 1991; Maad, 2000; Schiestl *et al.*, 2011; Schäffler *et al.*, 2012) or under artificial settings (e.g. Herrera, 2001; Aigner, 2004; Castellanos *et al.*, 2004).

Following the Grant–Stebbins model, a few subsequent studies presented clear connections between different pollinator assemblages and floral trait variation (e.g. Robertson and Wyatt, 1990; Johnson and Steiner, 1997; Moeller, 2005; Nattero and Cocucci, 2007; Anderson and Johnson, 2008; van der Niet *et al.*, 2014). For example, the study by Johnson and Steiner (1997) illustrated pollinator adaptation of spur length in the South African orchid *Disa draconis* complex to short- and long-tongued flies. Floral morphology diverged as plants became locally adapted to variation in the proboscis length, behaviour, colour preferences and flight period of the spatially separated fly species. Schlumpberger *et al.* (2009) documented a notable example of prominent variation in corolla lengths, nectar production and anthesis in the South American cactus species *Echinopsis ancistrophora*. Flowers with short corolla have morning anthesis and low nectar production, while longer corolla flowers have anthesis at dusk and abundant nectar. Populations with the longest corolla are also where sphingid moth pollination predominantly occurs, and pollination by solitary bees dominated the remaining populations with shorter corolla.

Most of these studies indicate local pollinator adaptation through correlational evidence between floral traits and pollinator differences. A further step is necessary to confirm that these differences in floral traits are pollinator-driven, and would act as a barrier to or reduce gene flow when the 'ecotypes' come into contact. In this study, we address this step through a reciprocal transfer experiment using the orchid species *Gymnadenia odoratissima*. Orchids are one of the most diverse plant families, with pollinator interactions considered to be the primary driving force of their diversification (Dressler, 1993; Schiestl and Schlüter, 2009; Harder and Johnson, 2009; Schiestl, 2012). Thus, orchids represent a significant model system for the investigation of pollinator adaptation. *G. odoratissima* is a nectar-rewarding species found throughout Europe, and is abundant in many calcareous regions of Switzerland where the study was conducted. The plant can inhabit lowland forests at around 500 m above sea level (m a.s.l) to subalpine meadows at up to 2600 m a.s.l. Thus, due to the large span in altitudinal gradient, it is likely that the pollinator assemblages differ in composition in lowland and mountain populations. It is known from previous studies that the pollinators of *G. odoratissima* are primarily Lepidoptera species (van der Cingel, 1995; Vöth, 2000; Huber *et al.*, 2005, and references therein), although the qualitative and quantitative variation in pollinator communities between the lowlands and mountains has not yet been defined. We characterized the pollinator community composition in the plant populations of the lowlands and mountains, and assessed any qualitative and quantitative differences between the pollinator communities. To investigate the existence of local pollinator adaptation, reciprocal transfers of cut plants were performed between lowland and mountain populations, and their pollination success compared with cut local plants was quantified. We predicted that if there is evidence of local adaptation, it would have resulted from spatially divergent evolution of floral traits. It has been shown that these traits may include display size (e.g. Galen, 1989), corolla dimensions (e.g. Nattero and Cocucci, 2007; Medel *et al.*, 2007; Anderson and Johnson, 2008; Gómez *et al.*, 2008; Martén-Rodríguez *et al.*, 2011; van der Niet *et al.*, 2014), spur length (e.g. Robertson and Wyatt, 1990; Johnson and Steiner, 1997; Anderson and Johnson, 2009; Peter and Johnson, 2014), floral scent (Mant *et al.*, 2005; Anderson *et al.*, 2009; Schiestl *et al.*, 2011; Parachnowitsch *et al.*, 2012; Peter and Johnson, 2014; van der Niet *et al.*, 2014), floral colour (e.g. Streisfeld and Kohn, 2007; Newman *et al.*, 2012) and nectar properties (e.g. Johnson and Nicolson, 2008; Schlumpberger *et al.*, 2009). Thus, we address the following questions: (1) Are there differences in the pollinator composition between lowland and mountain populations? (2) Do plants achieve lower reproductive success when transferred to a different altitudinal region compared with the local plants? (3) Are there differences in floral morphology, scent composition, colour and nectar sugar concentration between plants from the lowlands and mountains?

MATERIALS AND METHODS

Study species

Gymnadenia odoratissima (L.) L.C.M. Richard (Orchidaceae) is a terrestrial orchid species found in temperate and mountainous regions of Europe. The species has a flowering period generally from June to mid-August, with an overlap in flowering time between the lowland and mountain populations of approximately 3 weeks (M. Sun, pers. obs.). The plant inflorescences have between 10 and 100 flowers, with each flower producing nectar contained in a floral spur as food reward for pollinators. The pollination system is functionally specialized with visitations from diurnal and nocturnal Lepidoptera species (van der Cingel, 1995; Vöth, 2000; Huber *et al.*, 2005, and references therein). The flowers have colours ranging from deep pink to white, and emit strong floral scent during both the day and the night (Huber *et al.*, 2005).

Plant populations

Twelve populations (six lowland and six mountain populations) of *G. odoratissima* within Switzerland were sampled from June to mid-August between 2010 and 2012. Details of the geographical locations and the years when pollinator communities and floral traits (including sample sizes) were assessed, and transfer experiments were conducted are presented in Table S1 (Supplementary Information).

Pollinator observations

During the flowering period, pollinating insects were observed and caught from inflorescences of naturally growing plants throughout the day and evening. Insects observed (1) to probe the floral spur and feed from the nectar, (2) to have obtained pollinia or (3) to possess pollinia were classified as pollinators. These insects were caught using hand nets and individually stored in a −20 °C freezer. Commonly observed insect species, of which it was certain that the species had previously been caught, were recorded as observed but not caught. Pollinators observed from 0601 h to 1800 h were categorized as diurnal pollinators, and those observed from 1801 h to 0600 h were categorized as nocturnal. A total sampling time of 85·25 h (63·75 h during the day, 21·50 h during the night) was spent in the lowland populations, and 80·00 h (61·75 h during the day,

18·25 h during the night) in the mountain populations, calculated to the nearest 0·25 h. The number of pollinator observation hours was also used to calculate the pollinator visitation rate (number of pollinator observations per hour).

Transfer experiments

Two types of transfer experiments were conducted: vertical transfers and horizontal transfers. The vertical transfers consisted of bidirectional transfers of plants from lowland populations to mountain populations, and vice versa. The horizontal transfers were bidirectional transfers of plants between populations of the same altitude, i.e. between lowland populations and between mountain populations, as a control to test for population-specific effects on pollination success. Pollinia were removed from the experimental flowers to prevent gene pool contamination of the local populations. For each individual, any previously pollinated flowers were removed from the inflorescence, as well as any buds.

For the vertical transfers, a population of *G. odoratissima* at each of the two altitudinal levels was selected. In each population, 30 plants were randomly selected and cut at the stem at ground level. Within the 30 plants, 15 were placed in the population from which they were collected, referred to as 'local' individuals, and 15 were transferred to a population of the other altitudinal level, referred to as 'transferred' individuals. The 'local' individuals were used for comparison with the 'transferred' individuals from the other altitudinal level. In the lowland populations, the 15 'transferred' individuals were moved to the mountain population, and in the mountain populations, 15 'transferred' individuals were moved to the lowland population. The 'transferred' individuals were transported in plastic containers containing water and kept shaded throughout the transportation process. In each population, a series of 15 plots were set up along a transect, with each plot consisting of one 'local' and one 'transferred' individual. Each individual was placed in a 15-mL Falcon tube (BD, Franklin Lakes, NJ, USA) containing water, and set into the ground. The two individuals within a plot were placed approx. 20 cm apart, while the distances between the plots were approx. 2–5 m. It was ensured that the plots were at least 0·5 m from a natural neighbouring plant. This method of plant treatment does not negatively affect plant growth, as the plants were observed to develop, flower and set fruit under these conditions.

Horizontal transfers were performed in the same way as the vertical transfers, except that the 'transferred' individuals of a population were moved to another population of the same altitudinal level. The pairs of populations used in the vertical and horizontal transfers are listed in Table S2.

After a period of three weeks, all plots were collected. For each individual the number of pollinated flowers, the number of fruit capsules formed and the total number of intact flowers on the inflorescence during flowering were counted. From this, the proportionate female reproductive success (R_f) was determined for each individual using the following formula:

$R_f = (F_p + S)/F_i$

F_p is the total number of pollinated flowers, S is the total number of flowers that set fruits (fruit set) and F_i is the total number of flowers on the inflorescence. Both F_p and S were obtained to quantify female reproductive success, as the flowers on an inflorescence were at different developmental stages during plot collection. To ensure that pollinated flowers set fruit and thus can be used as a reliable measure of female reproductive success, a series of hand-pollination experiments were performed on 20 individuals in the lowland population 'Döttingen' and the mountain population 'Münstertal'. Fine-mesh wire cages were placed over each individual prior to plant flowering to exclude any pollinator visitations. During flowering, five flowers per individual were marked with coloured thread and hand-pollinated with one to two pairs of pollinia using wooden toothpicks. After three weeks, the pollinated flowers were examined for fruit capsule development.

Floral phenotype survey

Floral morphology measurement. We measured the length of inflorescences to the nearest centimetre (Fig. 1, left) by calculating the difference between plant height and stem length. Subsequently, two flowers per individual were sampled: a higher and a lower flower on the inflorescence. Flowers were stored in 2-mL Eppendorf tubes (Safe-Lock Tubes; Eppendorf AG, Hamburg, Germany) containing 70 % ethanol. In the lab, the individual flowers were placed in a clear Petri dish, and thinly immersed in a few drops of ethanol. The flowers were carefully spread out and flattened into position, facing down such that the spur and all the dimensions of the petals and sepals were entirely visible and fully extended. Photos were taken of each flower using a digital SLR camera (Nikon D90 D-SLR; Nikon Corporation, Tokyo, Japan) fitted with a 105-mm F/2·8D lens (AF-S VR Micro-Nikkor; Nikon Corporation), and attached to a fixed tripod.

Each photo was analysed using the image processing and analysis program ImageJ (http://rsbweb.nih.gov/ij/), with each floral image measurement calibrated to a 5-cm scale included. The floral traits numbered from 1 to 10 (Fig. 1, right) were measured for each flower. Flower shape (traits 1 and 2) and area (9), labellum size and shape (3, 4, 6, 7 and 10) and inflorescence size comprise the display signals for pollinator attraction, while spur length (8) affects nectar accessibility for potential pollinators. The mean value for each trait was calculated between the two flowers of each individual. The floral traits were also standardized to inflorescence size to test for any effects of resource limitation and trade-offs.

Floral scent collection and identification. Scent collection was performed during the day between 0800 and 1700 h, within the flowering period. The entire inflorescence of each individual was enclosed in oven bags (Nalophan; Kalle UK Ltd, Witham, UK) and sealed at the ends with twist close wires. Air was extracted from the bags using a battery-operated pump (PAS-500 personal air sampler Spectrex; Redwood city, CA, USA) for 30 min at a rate of 150 mL min^{-1}, through fine glass tubes containing approx. 20 mg of Tenax TA (80/100 mesh; Supelco, Bellefonte, PA, USA). In each population, the scent of the surrounding air was sampled under the same scent collection parameters, as a control. The glass tubes were sealed, transported to the lab and stored in a −25 °C freezer.

Analysis of the floral scent bouquet was conducted using gas chromatography with mass selective detection (GC-MS). Each glass tube was loaded and injected into the chromatograph

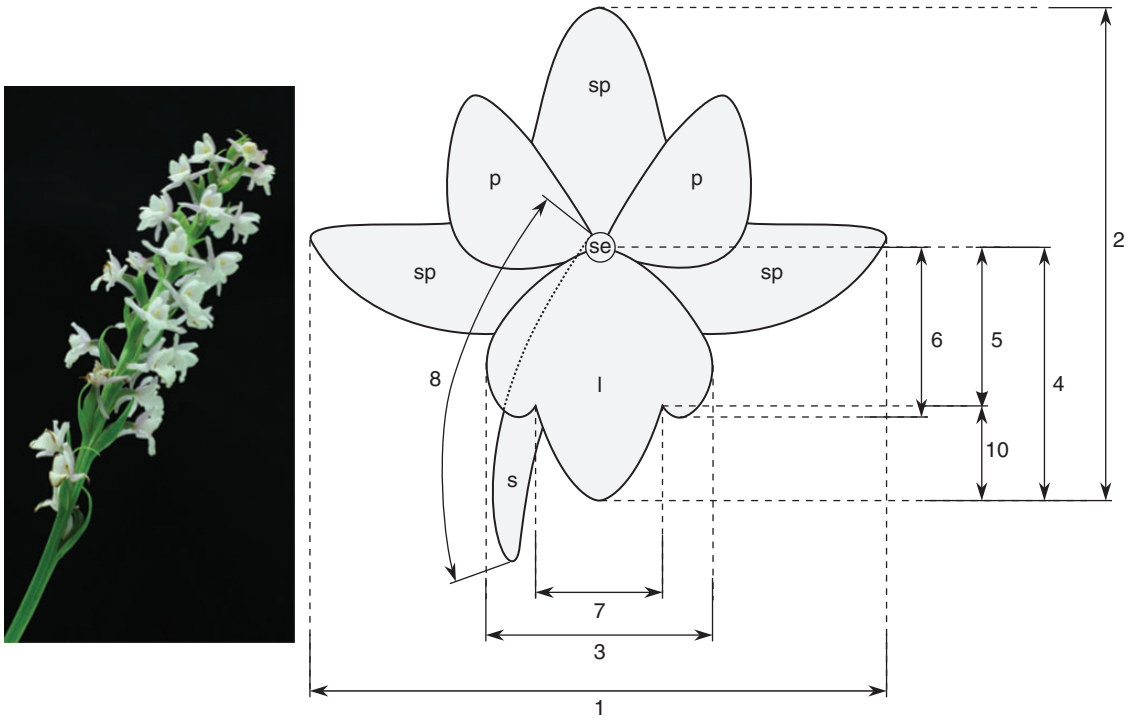

FIG. 1. *Gymnadenia odoratissima* inflorescence (left) and a diagrammatic flower (right) with morphological traits 1–10 indicated. Flower morphological traits are 1: flower width, 2: flower height, 3: labellum width, 4: labellum height, 5: spur entrance to height of interlobe, 6: side-lobe length, 7: interlobe distance, 8: spur length, 9: flower area (not shown, calculated by ImageJ from manually tracing the flower outline), 10: lobe length. Other floral traits indicated are p: petals, l: labellum, sp: sepals, s: spur, se: spur entrance.

(Agilent 6890 N) using a Gerstel thermal desorption system (TDS3, Gerstel, Mühlheim, Germany) with cold injection system (CIS; CIS4; Gerstel). For thermal desorption, the TDS was heated from 30 to 240 °C at a rate of 60 °C min^{-1} and held at the final temperature for 5 min. During the collecting of eluting compounds from the TDS, the CIS was set to –150 °C. For injection, the CIS was then heated to 250 °C at a rate of 12 °C s^{-1} and this temperature was held for 3 min. The gas chromatograph was equipped with an HP-5MS column (0·25 μm i.d., 0·32 mm film thickness, 30 m length), and helium was used as the carrier gas at 1·9 mL min^{-1} flow rate. Compound identification and quantification were achieved using a mass selective detector (Agilent MSD 5975). Chromatograms were analysed using the program ChemStation (G1701EA E.02·02 MSD Productivity ChemStation Software, Agilent Technologies, Germany). Preliminary identification of volatiles was done using the NIST spectral database implemented in the ChemStation program. Subsequently, retention times and mass spectrograms of all floral volatiles were compared with those of synthetic reference compounds. For quantification, calibration curves for qualifier ions were established for all compounds. To calculate the absolute amounts of floral volatiles, the peak areas of qualifier ions were converted into nanograms using the calibration curves. As the ChemStation program did not always correctly identify the peaks, all samples and compounds were manually double-checked and, if necessary, integrated manually. All absolute amounts were calculated as ng L^{-1} of sampled air. To exclude compounds produced in only trace amounts, a mean threshold of 0·5 ng L^{-1} air sampled per inflorescence was imposed, of which 22 compounds (for the list of compounds and their IUPAC name see Table S5) from the scent profile exceeded. We calculated the relative amounts for each of the 22 compounds separately by dividing the absolute amount of an individual compound by the sum of the absolute amounts of all compounds.

Floral colour measurement. Two flowers from each individual were sampled: one from the top and one from the lower part of the inflorescence. Each flower was wrapped in damp tissue paper, stored in a 1·5-mL Eppendorf tube (Safe-Lock Tubes) and kept in a 4 °C fridge until analysis. Flower labellum colour was measured as percentage reflectance using a AvaSpec-2048 Fibre Optic Spectrometer (Avantes B.V., Eerbeek, the Netherlands) and a AvaLight-XE xenon pulsed light source (Avantes B.V.). The fibre optic probe (Avantes B.V.) was held at a fixed distance and angle from the labellum using an enclosed fibre optic holder. The measurements were calibrated with a 98 % reflective polytetrafluoroethylene (PTFE) white and black reference tile (Avantes B.V.) at the beginning of each session of measurements. The reflectance spectra were expressed as a percentage of reflected light in relation to the white reference tile, with wavelengths between 350 nm (little to no reflectance was detected up to then) and 700 nm considered. All the equipment was connected to a laptop equipped with the data collection software AvaSoft 7·3 (AvaSoft-Basic, Avantes B.V.). Each reflectance spectrum was composed of 1206 percentage reflectance data points taken at 0·597-nm intervals. The mean percentage reflectance value at each reflectance interval was calculated between the two flowers of each individual.

Nectar sugar concentration measurement. All plants were placed under fine-mesh wire cages prior to flowering to exclude pollinators from influencing nectar volume and concentration. On the onset of anthesis, nectar was extracted from two flowers per inflorescence: from the uppermost and lowermost open flower. To measure the nectar sugar content, the spur was cut off from the flower as close to the spur entrance as possible using a pair of fine scissors. The nectar was carefully compressed out of the spur directly onto the optical glass of a hand-held refractometer (Eclipse 45-81, Bellingham & Stanley Ltd, Tubridge Wells, UK; 0–50 ° Brix units), and the sugar percentage (sucrose equivalent %) was read. The sugar percentage measurements from the two flowers of each individual were taken between 1100 and 1500 h for 20 marked individuals. This was done on the same individuals on two separate days in each population. The sugar percentage of each individual was determined from an average of the lower- and uppermost flower measurements for both days. To observe whether there were any effects of temperature and humidity on sugar concentration, for each sampled individual temperature and humidity data corresponding to the time of each measurement were taken from records at the nearest respective weather station: Beznau KKW (3·9 km from Döttingen), Ueken (4·7 km from Linn), Sta. Maria/Val Müstair (8·4 km from Münstertal) and Davos (1·1 km from Schatzalp).

Data analysis

To determine whether there was a significant difference in female reproductive success between 'local' and 'transferred' individuals, and whether the difference was affected by the altitude at which the population was situated, multiple logistic regressions were conducted. The proportionate female reproductive success was transformed into a binomial dataset (1 = pollinated, 0 = unpollinated) on an individual flower level for the conditional logistic (*clogistic*) model, to derive the success ratio for each plant individual. The *logit* link function was used in the model with 'treatment' (local/transferred) and 'altitude' (lowland/mountain) as the explanatory categorical variables. The computer software R (version 2·13·0, http://www.r-project.org/) was used for this analysis due to the suitability of the statistical package *Epi* (version 1·1·44, http://cran.r-project.org/package=Epi) for this dataset.

The following analyses were performed using IBM SPSS Statistics 20·0 (IBM SPSS, 2011). To test whether there were significant differences in morphology trait values and floral scent compounds between the populations and altitudes, and in which traits and compounds, generalized linear models (GLMs) were conducted separately for each morphological trait and scent compound, using 'population' nested within 'altitude' and 'altitude' as factors.

For floral colour comparison a principal component analysis (PCA) was performed to reduce the large number of values into a few orthogonal variables (principal components, PCs). The PCA was conducted with standardized values, using varimax rotation and extracting components with eigenvalues greater than 1. The PC scores were entered into two-way ANOVAs, examining whether there was significant difference in colour wavelength composition of individuals between populations and altitudes.

A four-way ANOVA was used to evaluate whether the variation in nectar sugar concentration was due to the altitudinal difference, or the abiotic factors humidity and temperature. In the model, sugar concentration was the response variable with the factors 'altitude', 'temperature' and 'humidity' as the explanatory variables.

RESULTS

Pollinator guilds in the lowland and mountains

The identification of all caught and observed pollinators (196 individuals in total) is reported only to the genus level, due to uncertain species-level identification of individuals of some genera (*Polyommatus*, *Adscita*, *Zygaena* and *Stenoptilia*). Likewise, one case was reduced to family level (Pterophoridae) and two cases to order level (Diptera and Coleoptera).

In the lowland populations, pollinators of two insect orders Lepidoptera and Coleoptera were observed (Fig. 2A). In the order Lepidoptera, a total of four butterfly families and four moth families were found, with the most frequent pollinators being the butterfly species *Ochlodes sylvanus* (Esp.) at 26·63 % of all pollinator visitations per hour and the moth *Phytometra viridaria* (Cl.) at 24·43 % of all pollinators h^{-1}. Neither of these pollinators visited plants in the mountains.

The pollinators observed in the mountain populations belonged to the three insect orders Lepidoptera, Diptera and Coleoptera (Fig. 2B). The order Lepidoptera was the most abundant, characterized by pollinators belonging to eight moth and three butterfly families. The most common pollinator was *Eudonia sudetica* (Z.), which comprised 28·65 % of all pollinators h^{-1} and was not observed to visit lowland plants. The functional group Diptera, which made up 8·47 % of all pollinators h^{-1} in the mountains, was also absent in the lowlands. Identification revealed that eight out of 11 Diptera specimens were species of the family Empididae.

The overlap of pollinator guilds between the lowland and mountain populations was minor, including individuals of the genus *Polyommatus* and the family Pterophoridae. The pollinators of the genus *Polyommatus* were one of the major pollinator groups of mountain flowers but made up only 6·14 % of the lowland pollinators h^{-1}. Pollinators of the family Pterophoridae made up 16·27 % of the mountain pollinators h^{-1}, but only 2·05 % of the lowland pollinators h^{-1}. In addition, species of the genus *Pieris* and the order Coleoptera were observed in both the lowlands and the mountains, but the visitation rate was very low at only one observation in each altitudinal region.

Nocturnal pollinators visited at a rate of 1·95 pollinators h^{-1} in the mountains, but at only 0·27 pollinators h^{-1} in the lowlands. The frequency of total pollinator observations in the mountains (1·96 pollinators h^{-1}) was approximately four times higher than that of the lowlands (0·46 pollinators h^{-1}).

Transfer experiment

For the vertical transfers, the reproductive success of lowland individuals was not significantly different whether they stayed in the lowlands or were transferred to the mountains ($z_{106} = 1·04$, $P = 0·299$; Fig. 3A), while the reproductive success of mountain individuals was significantly lowered when moved to the

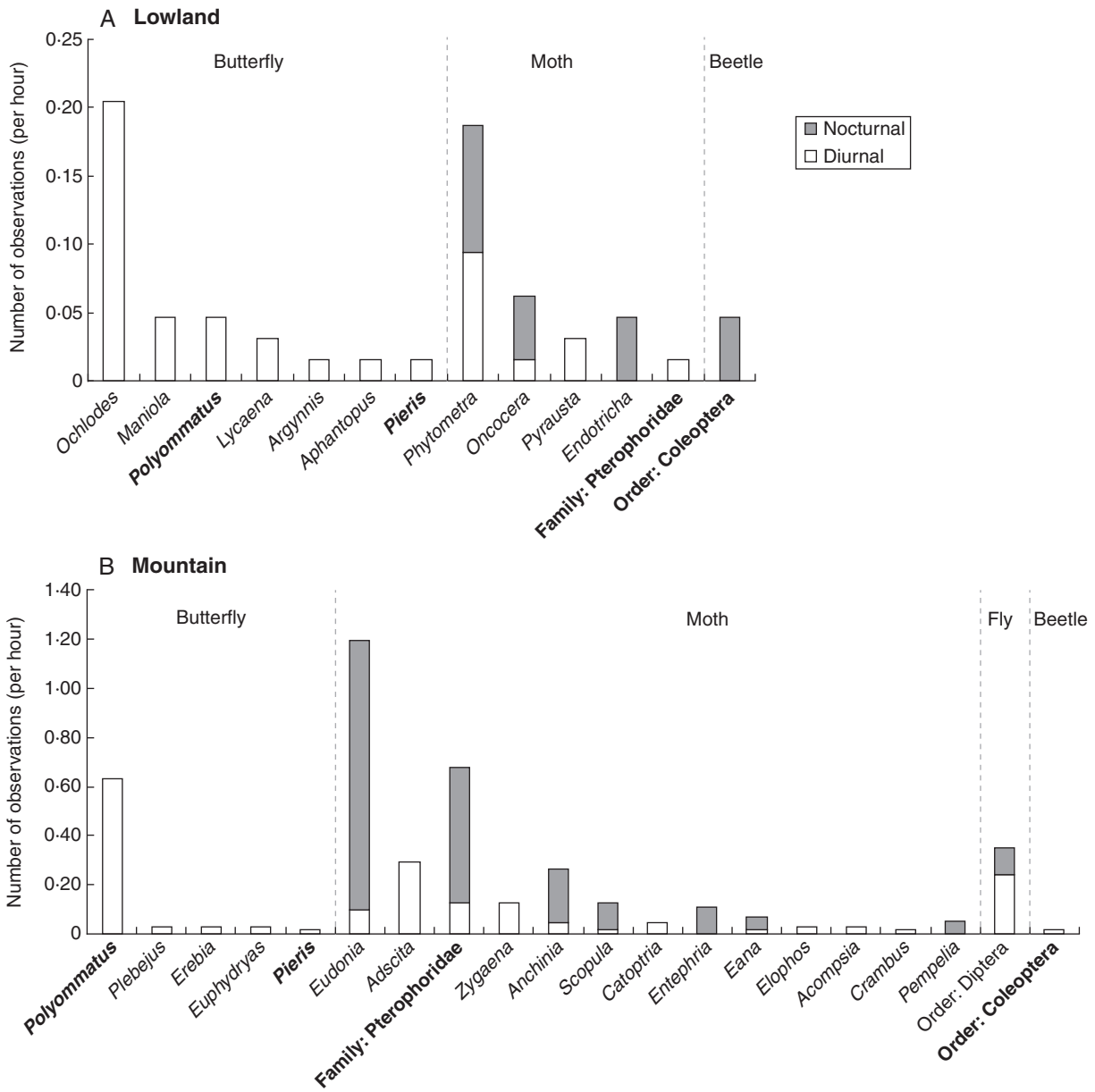

FIG. 2. The rate of pollinator individuals visiting *G. odoratissima* flowers measured as the number of pollinator observations per hour for (A) the lowland populations and (B) the mountain populations for each pollinator genus unless otherwise stated. The overlapping pollinators between the altitudinal regions are marked in bold.

lowlands compared with the left behind 'local' mountain individuals ($z_{107} = -4{\cdot}05$, $P < 0{\cdot}001$). Furthermore, there was a statistically significant difference between reproductive success of 'local' and 'transferred' individuals in both altitudinal regions. Mountain plants had consistently higher reproductive success than lowland plants. On the one hand, the mean female reproductive success for 'transferred' mountain individuals was significantly higher than that of the 'local' lowland individuals ($z_{127} = 5{\cdot}84$, $P < 0{\cdot}001$). On the other hand, the difference in mean reproductive success was also significantly greater for the 'local' mountain individuals, which received over three times higher reproductive success compared with the 'transferred' lowland individuals ($z_{86} = -8{\cdot}04$, $P < 0{\cdot}001$).

For the horizontal transfers, there was no significant difference in the reproductive success of lowland individuals between their 'local' populations and their 'transferred' populations ($z_{85} = 1{\cdot}90$, $P = 0{\cdot}057$; Fig. 3B), nor was there for mountain individuals ($z_{81} = 0{\cdot}80$, $P = 0{\cdot}421$). Moreover, there was no significant difference between the 'local' or 'transferred' individuals within the lowland populations ($z_{63} = 0{\cdot}95$, $P = 0{\cdot}342$) and the mountain populations ($z_{103} = 1{\cdot}48$, $P = 0{\cdot}139$). No significant difference was found between the reproductive success of the 'local' lowland and 'local' mountain individuals ($z_{82} = -0{\cdot}27$, $P = 0{\cdot}786$). There was no effect of the factor 'plot' on reproductive success in all populations ($z_{209} = -0{\cdot}22$, $P = 0{\cdot}826$).

In the hand-pollinated plants, there was no significant difference between the number of hand-pollinated flowers and the number of subsequent fruit set in both the lowland ($t_{18} = -1{\cdot}46$, $P = 0{\cdot}163$) and the mountain populations ($t_2 = -2$, $P = 0{\cdot}184$).

Floral morphology differences between altitudes

With regard to altitude differences, the mean morphological trait measurements of lowland flowers were all greater than those of the mountain flowers (Table 1). Flower width, labellum width, side lobe length and flower area were significantly larger in the lowlands. However, when the traits were standardized to the length of the inflorescence, all traits were significantly larger in the mountain populations than in the lowland populations (Table 1). Additionally, there were differences in absolute (Table S3 and Fig. S1) and standardized trait means among some populations (Table S4).

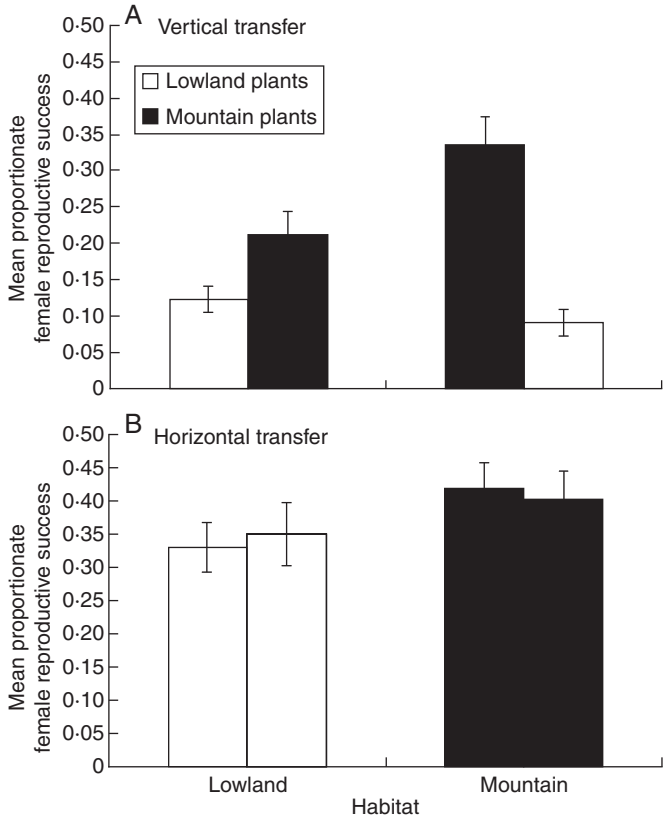

FIG. 3. The mean (± 1 s.e.) proportionate female reproductive success from (A) vertical transfers and (B) horizontal transfers in the lowland and mountain populations. For each pair of bars, left bar: 'local' individuals, right bar: 'transferred' individuals.

Floral scent differences between altitudes

The mean sum of the absolute amounts of compounds per inflorescence was higher in the lowlands (mean ± s.d. = 4392·75 ± 3776·79 ng L^{-1}) than in the mountains (3160·87 ± 2363·35 ng L^{-1}) ($t_{195·57} = 3·07$, $P = 0·002$). However, there was no difference in the mean amount of compounds emitted per flower between the lowland and the mountain populations ($t_{248·01} = -1·64$, $P = 0·1$).

There were significant differences in relative amounts for 18 of the 22 compounds in the scent emission between the altitudes (Fig. 4). There were significant population differences for all 22 compounds, apart from hexyl acetate and methyl eugenol (Table S6 and Fig. S2).When considering aromatic compounds and other compounds separately, the lowland populations had significantly higher relative amounts of nine out of 12 aromatic compounds compared with the mountain populations (Fig. 4A). By contrast, the mountain populations had significantly higher relative amounts of nine out of 10 non-aromatic compounds compared with the lowland populations (Fig. 4B).

Floral colour differences between altitudes

The PCA produced four PCs with eigenvalues greater than 1, explaining 99·79 % of the total variance in the data. PC1 (43·47 % of variance) had significant loadings of wavelengths between 488 and 636 nm, PC2 (22·44 %) of wavelengths between 400 and 487 nm, PC3 (17·08 %) of wavelengths between 637 and 700 nm, and PC4 (16·80 %) of wavelengths between 350 and 399 nm.

There was only a significant difference in the relative reflectance of wavelengths in PC1 between the lowlands and mountains

TABLE 1. *Mean (± s.d.) morphological trait values and standardized morphological trait values for flowers of the lowland and mountain populations*

		Mean trait value			Standardized mean trait value		
Trait	*n*	Lowland	Mountain	GLM altitude, z_1	Lowland	Mountain	GLM standardized altitude, z_1
(1) Flower width	231	9·13 ± 1·39	8·55 ± 1·12	29·54***	14·46 ± 3·60	18·16 ± 6·95	21·30***
(2) Flower height	231	7·73 ± 1·10	7·58 ± 0·91	2·47	12·18 ± 2·90	16·02 ± 6·03	31·25***
(3) Labellum width	229	3·37 ± 0·60	3·11 ± 0·54	22·49***	5·30 ± 1·28	6·52 ± 2·43	18·55***
(4) Labellum height	232	3·77 ± 0·56	3·67 ± 0·48	3·71	5·96 ± 1·47	7·73 ± 2·83	29·78***
(6) Side-lobe length	228	2·70 ± 0·49	2·56 ± 0·44	8·69**	4·26 ± 1·00	5·38 ± 2·05	23·25***
(7) Interlobe distance	226	1·69 ± 0·23	1·66 ± 0·26	1·49	2·72 ± 0·77	3·41 ± 1·10	27·66***
(8) Spur length	226	4·55 ± 0·52	4·54 ± 0·55	0·00	7·29 ± 2·05	9·58 ± 3·47	35·28***
(9) Flower area	227	31·36 ± 8·81	29·45 ± 6·98	7·14**	48·17 ± 13·66	61·72 ± 24·65	22·36***
(10) Lobe length	228	1·16 ± 0·27	1·18 ± 0·24	0·15	1·85 ± 0·60	2·45 ± 0·94	29·36***

All units of absolute trait values are in mm, apart from 'flower area' (trait 9) which is measured in mm^2. Data for 'spur entrance to height of interlobe' (trait 5) were removed prior to the analysis as it was a negligible trait used to derive trait 10 'lobe length'. Results from the generalized linear models of the mean absolute and standardized trait values are shown for trait comparisons between the altitudinal regions. Traits that are significantly different are shown as $*P < 0·05$, $**P < 0·001$, $***P < 0·0001$.

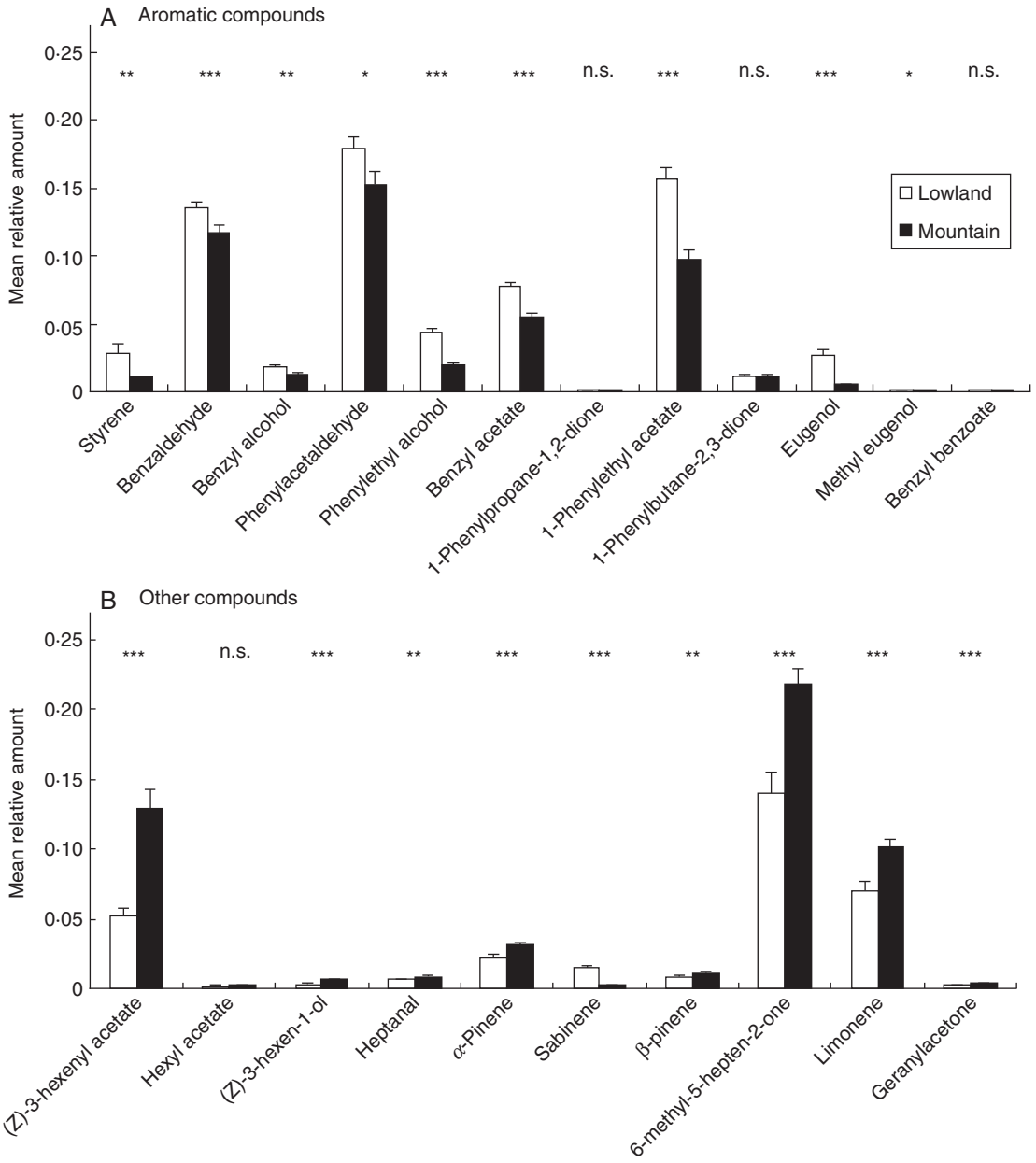

FIG. 4. The mean ($\pm$1 s.e.) relative amount of volatile scent compounds separated into (A) aromatic compounds and (B) other compounds, for individuals in the lowland and mountain populations ($n = 254$ for all compounds). The standard IUPAC chemical nomenclature of these compounds can be found in Table S5. Comparisons of the relative quantity of each compound were made between the two altitudinal regions using generalized linear models. Compounds with significantly different relative amounts between the altitudes are shown as $*P < 0{\cdot}05$, $**P < 0{\cdot}001$, $***P < 0{\cdot}0001$ above the bars.

($F_{1,6} = 33{\cdot}07$, $P = 0{\cdot}001$), as well as among some populations ($F_{7,156} = 32{\cdot}68$, $P < 0{\cdot}001$; Fig. S3). Field observations showed lowland flower colours to range from deep pink to pink, and mountain flowers as being comparably lighter with colours from light pink to white (M. Sun, pers. obs.).

Nectar sugar concentration difference between altitudes

The mean sugar percentage in the lowland populations 'Döttingen' (mean $\pm$ s.d. = 19·75 $\pm$ 3·04 %) and 'Linn' (15·95 $\pm$ 3·03 %) were slightly higher than that of the mountain populations 'Münstertal' (15·59 $\pm$ 2·22 %) and 'Schatzalp' (11·28 $\pm$ 1·91 %). A significant influence of altitude ($F_{1,158} = 15{\cdot}47$, $P < 0{\cdot}001$) and population ($F_{2,158} = 7{\cdot}98$, $P < 0{\cdot}001$) on the sugar percentage of the nectar was indicated. There was also a significant influence of temperature at the time of measurement on the nectar sugar percentage ($F_{1,158} = 15{\cdot}62$, $P = 0{\cdot}014$), although there was no evidence of any effect of humidity ($F_{1,158} = 0{\cdot}49$, $P = 0{\cdot}642$). Thus, we conclude that although nectar sugar concentration differed between the altitudinal regions, temperature is likely to be responsible for this difference as opposed to pollinators.

DISCUSSION

Although pollinator adaptation is often believed to be a driving force for the evolution of floral trait variation, few studies have explicitly tested this assumption by reciprocal transfer experiments. In this study, we investigated pollinator adaptation in the orchid *G. odoratissima* over a broad altitudinal range by conducting transfer experiments. The results showed local pollinator adaptation in mountain plants, and as well as distinct differences in pollinator guilds, floral morphology, scent composition and colour between lowland and mountain populations. Horizontal transfers within lowland and mountain regions did not show any significant differences in pollination success, eliminating the possibility of population-specific adaptation.

Local pollinator adaptation

Our reciprocal transfer experiment strongly suggests that the observed differences in pollination success were caused by the different abilities of plants to attract pollinators in non-native regions, or by 'local' (native) pollinators depositing pollinia onto stigmas of 'transferred' (non-native) plants with lower efficiency (not measured here). Although the method of using cut plants for this experiment may alter the plant floral scent (Schiestl *et al.*, 1997), such physiological changes would have occurred in both the 'local' and the 'transferred' plants. As both these plant groups were subjected to the same treatment, there should not have been any systematic bias in the experiment.

We suggest that our transfer experiment results can be explained by considering the lowland and mountain plants as specific pollination ecotypes. Evidence for these altitude-specific ecotypes can be derived from observing the pollination success of plants moved to a non-native region. We found that the reproductive success of the lowland ecotype was not significantly different when they were moved to the mountain environment compared with their reproductive success in their 'local' lowland environment. Conversely, when the mountain ecotype was moved to the lowlands, it suffered a loss in reproductive success. As the reproductive success of the natural lowland plants and natural mountain plants are not significantly different, it cannot be said that the reduction in the reproductive success of the 'transferred' mountain plants was due to the lower pollinator visitation frequency in the lowlands. Rather, it could be explained in terms of the adaptation of mountain ecotypes to a relatively abundant functional group of pollinators that was not observed in the lowlands, namely the empidid flies. The absence of pollinating empidids may explain the decrease in reproductive success of mountain plants in the lowlands. Pollination by flies has been established before to be more abundant at higher altitudes (Arroyo *et al.*, 1982) and of greater importance in mountain plants compared with lowland plants (Müller, 1881; Dressler, 1993; Mani and Giddings, 1980), as they are thought to increase in importance in cooler climatic conditions (Faegri and van der Pijl, 1979; Warren *et al.*, 1988). Empidid flies are primarily predatory but their mouthpart is thought also to be well suited for extracting nectar from flowers with medium spur or corolla lengths. Empidid flies have previously been recorded as orchid pollinators, in particular as frequent diurnal visitors of *G. conopsea*, the sister species of *G. odoratissima*, in Norway (Sletvold *et al.*, 2012). The orchid *Platanthera stricta*, occurring in subalpine forests, is also thought to be pollinated by empidids (Patt *et al.*, 1989).

Pollinator guilds and floral trait differences

We observed significant differences between the lowland and mountain populations in the majority of the floral traits measured, together with pronounced differences in the pollinator communities. Aside from pollinator interactions, other biotic factors can influence floral and plant trait evolution, such as herbivores (Gómez and Zamora, 2000; Gómez, 2003; Strauss *et al.*, 2004), seed predators (Cariveau *et al.*, 2004; Carlson and Holsinger, 2010), nectar robbers (Galen and Cuba, 2001; Irwin *et al.*, 2001; Galen and Butchart, 2003) and competitors (Levin and Brack, 1995). Additionally, there are abiotic factors such as the environment and climate which may impact floral traits through, for example, drought (Galen, 2000) and heat stress (Coberly and Rausher, 2003). Our reciprocal transfer experiments, however, clearly document floral adaptations due to the different ability of plants to achieve pollination success through pollinator attraction. Herbivores or abiotic factors were not observed to cause any detrimental effects on the experimental plants. It is, however, unlikely that all measured traits contribute equally to pollinator adaptation, and thus the observed differences may represent an adaptive compromise to selection by pollinating and non-pollinating (biotic and abiotic) agents over a geographical area (as reviewed by Gómez and Zamora, 2000; Strauss and Whittall, 2006; Cosacov *et al.*, 2014).

One of the most pronounced discrepancies between the two altitudinal regions was the existence and quantity of empidid flies as pollinators in the mountains. These flies may impose different selection as compared with lepidopterans, due to their considerably different morphology and possible disparities in preference for floral signals. Floral scent was thought to be the primary attractant of empidid flies in *Plathanthera stricta*, where bioassays have shown that without a visual stimulus of the flower, the floral scent will elicit probing behaviour in these insects (Patt *et al.*, 1989). The scent compounds that *P. stricta* has in common with *G. odoratissima* are α-pinene, benzaldehyde, β-pinene, limonene, benzyl alcohol and phenylethyl alcohol (Patt *et al.*, 1988). Our results showed that half of these compounds (α-pinene, β-pinene and limonene) were more abundant in the mountain populations compared with the lowlands. However, further investigations are needed to understand more about potential selection by empidids on specific floral scent compounds, as well as on floral colour and morphology in the mountain *G. odoratissima.*

Besides the prevalence of empidids, we also noted the existence of more moth compared with butterfly pollinators in the mountain populations, in addition to qualitatively and quantitatively more nocturnal pollination. Most of these nocturnal pollinators belonged to the families Geometridae and Pyralidae, which are species operating predominantly at dawn or dusk and at night during warm summer weather (Willmer, 2011). Moth pollination is generally associated with plants with paler shades of floral colour, compared with the broader colour ranges of butterfly-pollinated flowers. Many studies have found that moth-pollinated species visited flowers that are white, cream or yellow (e.g. Oliveira *et al.*, 2004). Our observations are consistent with these results as flowers in the mountains were considerably lighter, in

contrast to lowland flowers. These light-coloured flowers could permit nocturnal pollinators to visually discern them more easily under very low light conditions. It has been documented that moth preferences switch from pink and yellow flowers in the early evening to exclusively white flowers in the night (Schremmer, 1941). However, establishing the location of flowers often required the aid of strong, sweet scent (Klahre *et al.*, 2011). We showed here that alpine plants emit relatively more non-aromatic compounds, while lowland populations emit greater relative amounts of most aromatic compounds. These differences could be due to dissimilar preferences of the pollinator communities. Soil nutrients may also play an important role, as most aromatic compounds analysed here are synthesized from phenylalanine as a start substrate (Dudareva *et al.*, 2013). While nitrogen is required for amino acid synthesis in plants, it is generally known that alpine plant productivity is constrained by the limited supply of nitrogen in mountain soil compared with the lowlands (Lütz, 2012). Thus, nitrogen limitation may explain some of the altitudinal differences in floral scent bouquets.

Apart from colour and scent, floral morphology is also a key trait for pollinator adaptation. Although flower dimensions were found to be larger in lowland plants, standardized trait values indicated that mountain flowers were significantly larger relative to their inflorescence size for all traits compared with the lowland flowers. This shows that alpine populations may allocate relatively more resources to display size, perhaps to compensate for the shorter flowering period in the mountains.

Implications of the study

The differences in plant traits between the altitudes are consistent with the hypothesis initially proposed by Grant and Grant (1965) and Stebbins (1970) that divergence in floral form is attributed to the variation in geographical pollinator mosaics. Our results agree with previous reports supporting this theory, such as a study by Miller (1981) which suggested that differentiation of flower colour and spur length in three geographically separated populations of *Aquilegia caerulea* is caused by differences in composition and abundance of hawkmoth species. Floral variation over different islands was shown by Martén-Rodríguez *et al.* (2011), where divergence in *Heliconia bihai* between two islands corresponded to differences in pollinators on the islands. Our study takes a further step from correlating floral traits with pollinator differences by confirming that trait differentiation are pollinator-driven through transfer experiments.

To better understand which traits underlie pollinator adaptations in plants, future studies should explore patterns of phenotypic selection on floral traits in different populations and regions. Furthermore, the molecular basis of adaptive traits, as well as the variability of adaptive genes in natural populations, needs to be investigated to improve our understanding of how patterns of variability allow adaptations to fluctuating pollinator environments. Such organismal and molecular micro-evolutionary studies may present vital contributions to understanding the processes of plant evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1. Geographical locations of the lowland and mountain populations of *G. odoratissima* within Switzerland, the year of pollinator observations and transfer experiments, and the year and sample size for floral phenotype measurements in each population. Table S2. Mean proportionate female reproductive success in each of the lowland and mountain populations used in the vertical and horizontal transfer experiments. Table S3. Mean of morphology trait values for all populations of the lowland and mountains. Fig. S1. Generalized linear model comparisons with Bonferroni post-hoc tests between all pairs of populations for each of the nine morphological traits. Fig. S2. Generalized linear model comparisons with Bonferroni post-hoc tests between all population pairs for each of the 22 scent compounds. Fig. S3. ANOVA comparisons with Bonferroni post-hoc test between all population pairs for PC1 from the floral colour PCA.

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