THE POLLINATION OF *EPIPACTIS* ZINN, 1757 (ORCHIDACEAE) SPECIES IN CENTRAL EUROPE – THE SIGNIFICANCE OF CHEMICAL ATTRACTANTS, FLORAL MORPHOLOGY AND CONCOMITANT INSECTS

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ABSTRACT

A series of studies on pollination biology of the *Epipactis atrorubens* (Hoffm.) Besser, *E. purpurata* Sm., and *E. palustris* (L.) Crantz populations was conducted in Poland, Lithuania and Czech Republic between 2003 and 2010. The research focused on pollinators and visitors to aforementioned orchid species as well as on the chemical analysis of orchids' nectar which was done using the GC/MS method. It was found that: 1) the type of pollinators depends on the orchid population size and the surrounding environment, where the temperature and amount of precipitation during the vegetative season are the most vital factors; 2) pollinators and visitors to the examined orchids might differ in successive growing seasons; 3) the studied *Epipactis* species differ in the chemical composition of their nectar and its scent, which can influence their pollination biology; 4) the tendency to autogamy observed in *E. purpurata* might be due to lack of pollinators in its habitats.

KEY WORDS: *Epipactis*, autogamy, geitonogamy, pollinators, vectors, orchids, nectar composition, GC/MS.

INTRODUCTION

Despite an ongoing research, the collected data on *Epipactis* differ significantly, and their breeding system still remains unidentified. As stated by Neal and Anderson (2005), the breeding system is most likely a consequence of a relatively short existence of Orchidaceae as a species, as well as its remarkable adaptability. Additionally, the chemical composition of nectar in the species of *Epipactis* has not been defined and there is no certainty whether it contains compounds directly responsible for attracting insects.

The Helleborines are rhizomatous orchids with a tendency towards autogamy. Yet, some literature indicates cases of geitonogamy, as well as allogamy and predominantly allogamy (Tałałaj and Brzosko 2008). Autogamic or facultative autogamic species also arouse controversy around their pollination ecology. It is still not clear whether autogamy results from lack of pollinators, ineffective pollination or is caused by other factors.

Although biology and ecology of orchids, their pollination mechanisms, their nectar composition and its functions, along with information on the genus *Epipactis* are often described, the data are frequently inconsistent (Nilsson 1978; Brantjes 1981; Cingel 2001; Jakubska et al. 2005a, b; Jakubska-Busse and Kadej 2006, 2008).

The study was designed to test whether autogamy in Helleborine is caused by lack of pollinators or whether it depends on specific insects species' that pollinate, how often the insects visit the flowers, and whether the flower's scent is a factor in the luring process. Additionally, nectar composition and the causes of geitonogamy were examined.

MATERIAL AND METHODS

An extensive investigation of pollination biology and ecology of Helleborine was conducted on the following populations (plant communities differing in size, origin, and location) between 2003 and 2010:

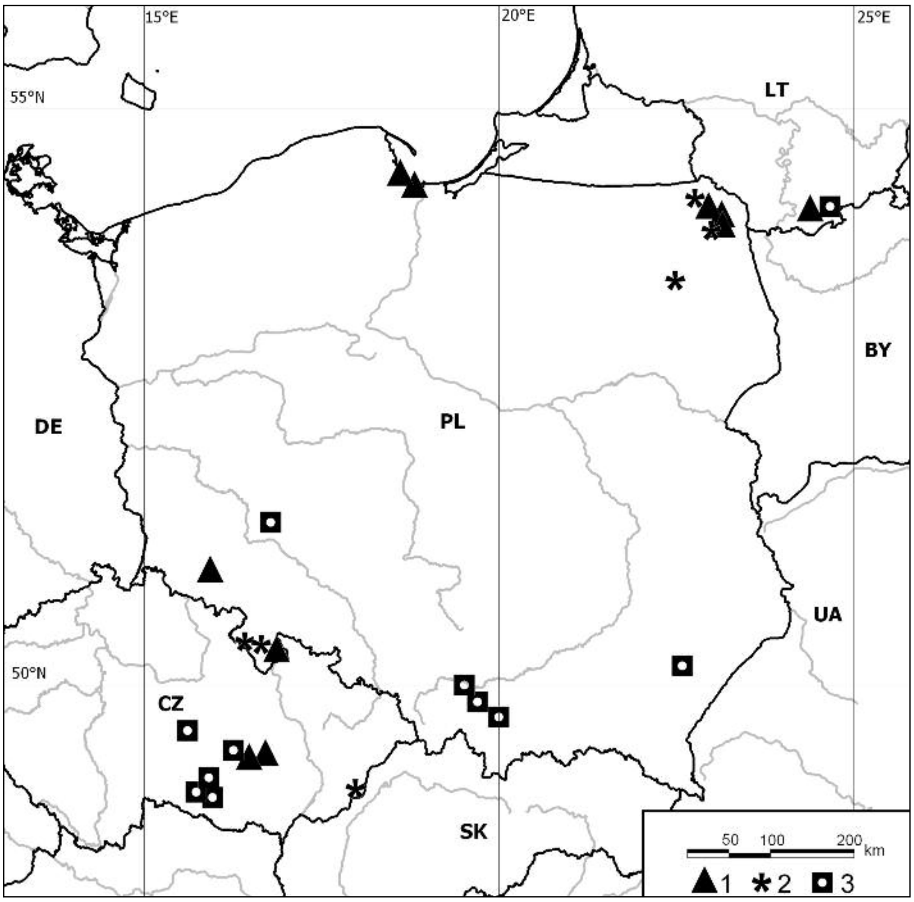


Fig. 1. Map of distribution of species studied in Poland, Czech Republic and Lithuania: **1** – *Epipactis atrorubens* (Hoffm.) Besser; **2** – *Epipactis palustris* (L.) Crantz; **3** – *Epipactis purpurata* Sm.

E. atrorubens (Hoffm.) Besser: Poland: Sopot, Gdańsk-Sobieszewo, Kopanica (Augustowszczyzna), the environs of Rowy (Suwalszczyzna), Wigry National Park, Połom (Kaczawskie Mountains), Kletno, the Czech Republic: Čebín, Moravský Kras, Lithuania: Merkinė, Dzūkija National Park.

Epipactis palustris (L.) Crantz: Poland: Turtul, Malesowizna (Suwałki district), Polskie Wrota Pass near Duszniki Zdrój, Stary Waliszów near Kłodzko, Biebrzański National Park, the environs of Kopanica village (Augustów lake district), the Czech Republic: Bílé Karpaty.

Epipactis purpurata Sm.: Poland: Straża (environs of Wińsko), Pogórze Cieszyńskie: the environs of Spytkowice, Kalwaria Zebrzydowska, Pcim and Sieniawa), Lithuania: – the environs of Vilnius, and the Czech Republic: South Moravia, the environs of Jihlava: Nove Syrovice, Kravsko, Vranovská Ves, Popůvky, Tetčice, Olbramkostel (see Fig. 1).

Insects behaviour

The insects were observed and photographed in natural conditions. In order to track insects' activity, the inside of the flower was marked with fluorescent pigments Radiant colour N.V. type TP33 (orange) and TP10 (chartreuse).

In order to determine the size of autogamy, cotton nets were put on selected inflorescences in specific populations well before the flowering period, which made the flowers inaccessible to pollinators. Next, the number of fruit produced by the plants was recorded in August.

Chemical analyses

Nectar was collected from flowers by using capillary glass with methylene chloride (GC Grade Merck) or n-pentane (GC Grade, Merck) as eluents, and stored in glass vials.

Then both extracts were condensed to 1ml volume using a rotary vacuum evaporator. The samples were analyzed using Perkin Elmer Gold TurboMass spectrometer with electron impact ionization EI and Perkin Elmer Autosysytem XL gas chromatograph equipped with an autosampler. The injector temperature was 320°C.

An Elite 5 MS column (5% phenylpolysiloxane) was used for the analyses (30 m long, inner diameter 0.25 mm, film thickness 0.25 μm, Perkin Elmer). Helium was used as the carrier gas at a flow rate of 0.8 ml/min, which was maintained by an electronic pneumatic control.

The GC oven temperature was held for 5 min at 40°C, then increased by 12°C/min to 300°C and held for 5 min. The temperature of MS transfer line was 250°C and the ion source worked at 180°C. The mass spectra were taken at electron energy 70 eV, with a scanning speed of 1 scans per 0.1 s from m/z 20 to 400.

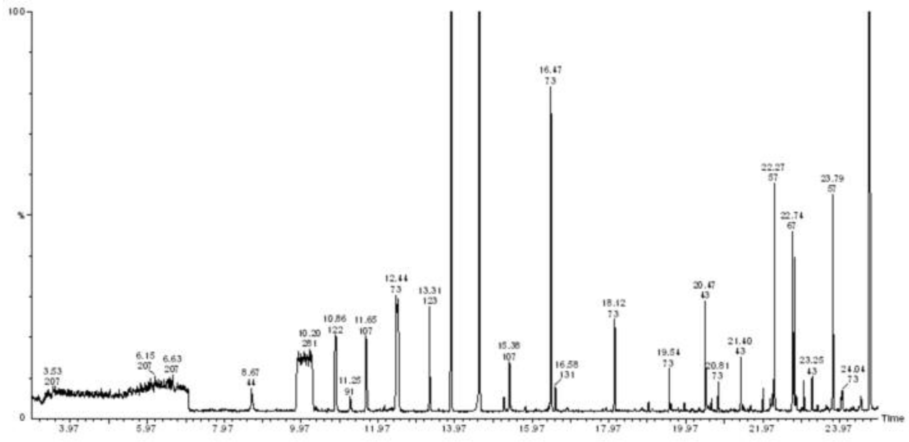
The GC-MS data were processed using the TurboMass Software v. 5.4.2. Component identification was carried out using the NIST 2.1.0, NBS and WILEY mass spectral database.

Each analysis was performed in triplicate to assess the reproducibility of results.

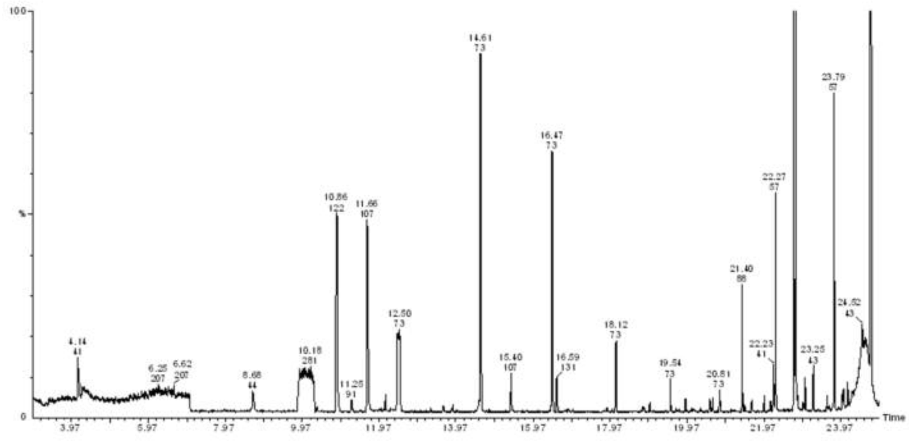
The analysis of nectar chemical composition was done with consent from the Minister of the Environment, no. DONOOŚogiz-4211/I-6/129/08/ep.

Field study

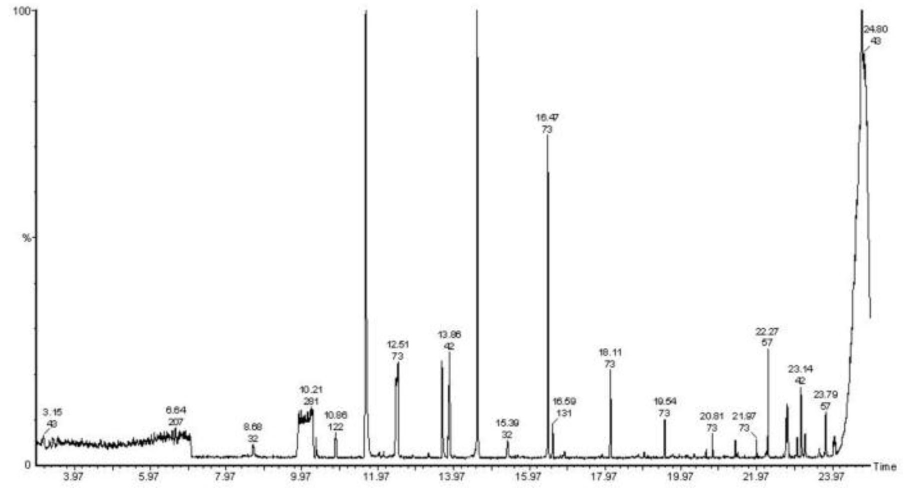
The experiment was performed in field conditions. In order to test how insects react to flower morphology, colour pictures of the examined species were placed in



A – *Epipactis atrorubens* (Hoffm.) Besser



B – *Epipactis palustris* (L.) Crantz



C – *Epipactis purpurata* Sm.

Fig. 2. Total Ion Current (TIC) chromatograms of Helleborine's nectar chemical composition.

close proximity to the studied ramets. This procedure allowed for determining the significance of flower physical qualities (such as its colour and shape) versus chemical attractants secreted by orchids in luring the insects.

RESULTS AND DISCUSSION

Nectar chemical analyses

The conducted analyses exposed qualitative and quantitative differences in nectar chemical composition of the examined species. Key attractants responsible for luring insects are: vanillin derivatives, e.g. 3,4 dimethoxybenaldehyde, or 1-methoxy-4-methyl benzene, octanal, nonanal, decanal. *E. atrorubens* nectar also contains compounds not found in other species: -2-methoxy-4-methylphenol; -3,4--dimethoxytoluen; -bis-(3,5,5-trimethylhexyl) ether.

Marsh Helleborine's nectar is much richer in aromatic compounds than that of Violet Helleborine. Among other things, it contains 2-decen-1-ol and numerous esters, e.g. methyl ester 11,14,17-eicosatrienoic amid, methyl ester 9,12-octadecanoic acid. All examined species produce nectar containing alcohols, e.g. 1-tetradecanol, 1-eicisanol, 2--decen-1-ol, 2-octen-1-ol, benzeneethanol, benzenemet-

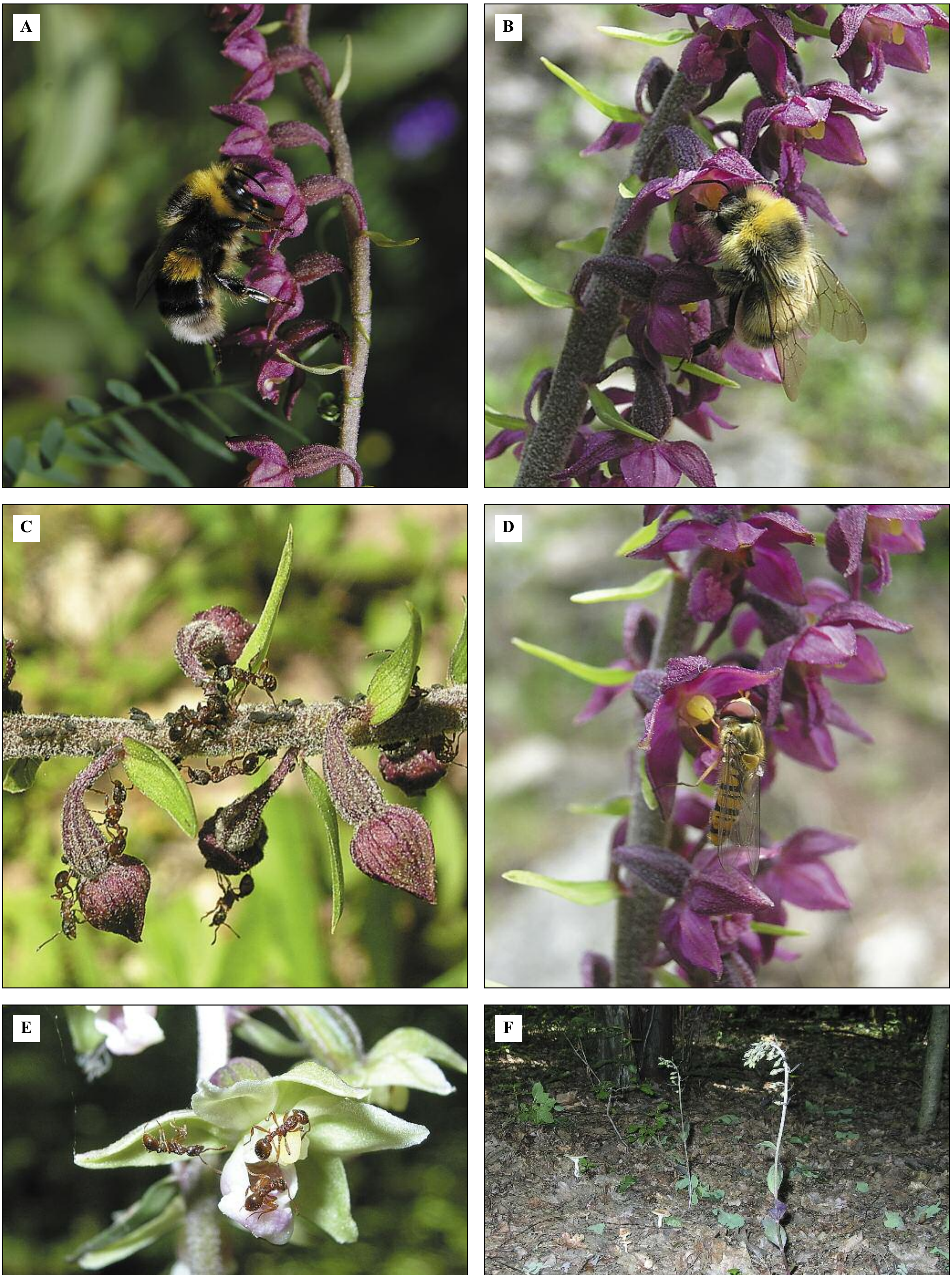


Fig. 3. **A** – male *of Bombus hortorum* (Linnaeus, 1761) (Hymenoptera, Apidae) on *Epipactis atrorubens*; **B** – male of *Bombus lucorum* (Linnaeus, 1758) (Hymenoptera, Apidae) *E. atrorubens*; **C** – *Myrmica rubra* (Linnaeus, 1758) (Hymenoptera, Formicidae) and aphids on *E. atrorubens*; **D** – *Episyrphus balteatus* (De Geer, 1776) (Diptera, Syrphidae) on *E. atrorubens*; **E** – *Myrmica rubra* (Linnaeus, 1758) (Hymenoptera, Formicidae) on *E. purpurata*; **F** – *Epipactis purpurata* · the habitat of the species.

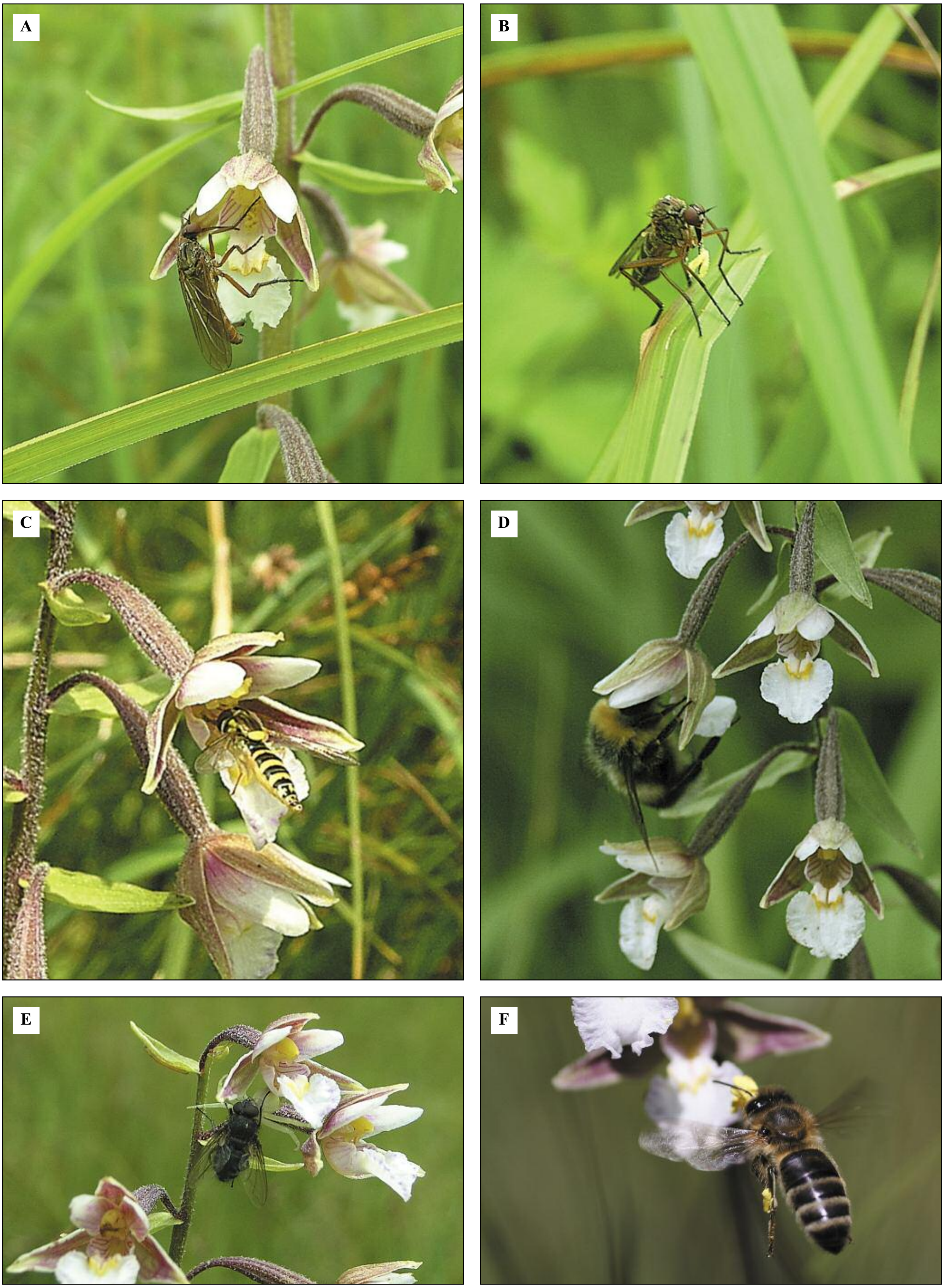


Fig. 4. **A** – *Asilus* sp. (Diptera, Asilidae) on *E. palustris*; **B** – *Asilus* sp. (Diptera, Asilidae) with pollinium packet of *E.pallustris*; **C** – *Episyrphus sp.* (Diptera, Syrphidae) on *E. palustris*; **D** – *Bombus hortorum* (Linnaeus, 1761) (Hymenoptera, Apidae) on *E. palustris*; **E** – Diptera on *E. palustris*; **F** – *Apis mellifera* Linnaeus, 1758 (Hymenoptera, Apidae) on *E. palustris*.

hanol, which confirms occurrence of substances attractive to Vespidae. The main attractants found in Marsh Helleborine's nectar are: nonanal (pelargonaldehyde), decanal, eicosanol and its derivatives.

Figure 2 shows a list of chromatograms TIC GC/MS.

Surprisingly, *E. atrorubens* and *E. palustris* nectars have scents similar to each other but different from that of *E. purpurata*. It is possible that vanillin and its derivatives overwhelm other compounds constituting the scent (see Fig. 2).

Pollinators and visitors

According to Cingel (2001), the most important pollinator of *Epipactis* species found in Europe is *Dolichovespula* sp. (Hymenoptera: Vespidae). Other pollinators include *Halictus* bees, honeybees, and hoverflies. Additionally, *Vespula arenaria*, *V. consobrina*, *V. vidua*, and *Polises fuscatus* (Hymenoptera: Vespidae) (Cingel 2001) might successfully pollinate *Epipactis* orchids.

The results only confirm that species of *Epipactis* in Central Europe are pollinated mainly by Vespidae and Apidae.

A strong tendency towards autogamy in small populations or populations which have a limited access to pollinators (e.g. dark forests) was found. In open populations such tendency was not found. The results partially confirm the Burns-Balogh et al. (1987) hypothesis that "autogamy is a pre-adaptation towards colonizing new habitats".

Another hypothesis stating that ants might be effective pollinators of *Epipactis* species has not been proven. Ants, mentioned by many authors (e.g. Peakall and Beattie 1989) as pollinators, rarely participate in pollination and should rather be treated as a potential vector. In Central Europe, the presence of ants, as well as their minute size, should rather be related to collecting the pollen as a food source. They are also drawn to orchids because of the nectar or honeydew produced by aphids living on orchids' shoots, both gathered for nourishment (see Fig. 3C and E). Ants, however, are main pollinators of tropical orchids such as *Leporella fimbriata* (Orchidaceae), the Australian self-compatible orchid that is pollinated by winged male ants (*Myrmecia urgens*), which are sexually attracted to the flower and pseudocopulate with it (Rico-Gray and Oliveira 2007). Another Australian orchid, *Microtis parviflora*, is pollinated by flightless worker ants of the *Iridomyrmex gracilis* species complex (Peakall and Beattie 1989).

According to the literature, among the studied orchid species, *E. palustris* is visited by insects most frequently. The number of its potential pollinators is estimated at 103 species (Nilsson 1978). The main pollinators of *Epipactis palustris* are mostly nectar feeders, such as flies, honeybees and digger wasps (Pijl and Dodson 1966). According to Nilsson (1978), Marsh Helleborine is pollinated mostly by nectar feeding solitary wasps (Hymenoptera: Eumenidae) as well as by ants, bumblebees and hoverflies. Brantjes (1981) found that Syrphidae (Diptera) and *Myrmica* sp. (Hymenoptera: Formicidae) also belong to *E. palustris* pollinators. Proházka and Velísek (1983) reported that *Sacrophaga carnaria* (Diptera: Sacrophagidae) and *Coelopa frigida* (Diptera: Coelopidae) pollinated Marsh Helleborine. Other sources also point to Vespoids as the pollinators of *Epipactis* species (Cingel 2001).

The study has shown that pollination biology varies greatly depending on the environmental factors as the air temperature and the amount of precipitation in the flowering season greatly influenced the results (Jakubska-Busse and Kadej 2008). Consecutive studies conducted on the same population over the course of several seasons revealed considerable differences in the quantity and the quality of pollinators. These differences are attributed to weather conditions.

The observations recorded during the study point to Diptera (*Empis* sp., *Episyrphus* sp.) and Hymenoptera (*Apis mellifera*) as the main pollinators of *Epipactis palustris* (see Fig. 4). It is likely that the numerously noted *Calyptrata* sp. along with other Diptera could also be pollinators. The presence of *Meligethes aeneus* (Coleoptera: Nitidulidae) should not, however, be directly related to pollinating *Epipactis palustris* due to a small body size of these beetles (Jakubska et al. 2005b).

The long list of insects (see Table 1) that have been found to frequently visits the flowers as well as the rich in attractants nectar composition (see Fig. 2) suggest that autogamy in this species is not caused by lack of potential pollinators or a poor luring strategy.

To test the range of autogamy, i.e. recurring visits to the same flower, and geitonogamy, (recurring visits to the same inflorescence), the flowers' inside of chosen specimens of *E. atrorubens*, *E. palustris* and *E. purpurata* was covered with fluorescent pigments to colour their visitors, which then allowed tracking their trajectory and behaviour. The insects prefer shoots with fresh flowers and visit them frequently which points to nectar consistency and intensity of secreted fragrance as strong attractants. The withering flowers or ones with dried nectar are far less attractive to insects and thus less often visited. The marked insects were also followed and observed on other plant species neighbouring the orchids, e.g. *Campanula rapunculoides* (Campanulaceae) and *Chamaenerion angustifolium* (Onagraceae). Some insects, including *Bombus* sp., walk along the inflorescence, starting on the bottom and moving upwards. They usually visit several flowers and repeat that pattern regardless of the plant species they visit. In conclusion, geitonogamy results from pollinators' biology (mainly Vespidae and Apidae), and is not related to Helleborine floral structure.

In open populations of all studied taxa, where other floral plants co-occur with orchids and serve as an alternative source of food for pollinators, autogamy was relatively rare.

Similar results were observed in the studies on pollination biology of *E. atrorubens*. The species was much often visited by insects during a warm and dry summer than during a humid one, which is due to the fact that insects do not fly in the rain, and that the emission of attractants is limited during rainfall and at low temperatures.

Since insects are poikilotherms and their activity depends on ambient temperature, *Bombus* sp. appears to be the main pollinator of Royal Helleborine (see Fig. 3). We found that bumblebees appeared on flowers the earliest with visits peaking between 10 a.m. and 2 p.m.

It confirms that they fly out to feed at approximately 10°C, and thus they are able to pollinate flowers during cold and rainy seasons. Bumblebees are more effective and more hardworking than honeybees. The high frequency of bumblebee's visits to orchids mentioned in other published research reports can be attributed by this bio-physiological adaptation.

TABLE 1. The main pollinators of selected species of *Epipactis* Zinn, 1757 in comparison to *Epipactis helleborine* (L.) Crantz.

Species of *Epipactis*	List of pollinators and concomitant insects	References
Epipactis helleborine (L.) Crantz	Wasps	Darwin 1877; Knuth 1909; Proctor and Yeo 1973
	HEMIPTERA: HOMOPTERA *Anaspis frontalis* (Linnaeus, 1758) (Scraptiidae) COLEOPTERA *Rhagonycha fulva* (Scopoli, 1763) (Cantharidae) *Coccinella septempunctata* Linnaeus, 1758 (Coccinellidae) *Propylea quatuordecimpunctata* (Linnaeus, 1758) (Coccinellidae) *Meligethes aeneus* (Fabricius, 1775) (Nitidulidae) *Lagria hirta* (Linnaeus, 1758) (Lagriidae) *Malachius bipustulatus* (Linnaeus, 1758) (Malachiidae) HYMENOPTERA: ACULEATA *Ichneumon* sp. (Ichneumonidae) *Myrmica ruginodis* Nylander, 1846 (Formicidae) *Vespula germanica* (Fabricius, 1793) (Vespidae) *Vespula vulgaris* (Linnaeus, 1758) (Vespidae) *Polistes gallicus* (Linnaues, 1767) (Vespidae) *Colletes daviesanus* Smith, 1846 (Vespidae) *Apis mellifera* Linnaeus, 1758 (Apidae) *Psithyrus bohemicus* (Seidl, 1837) (Apidae) *Lasioglossum fratellum* (Pérez, 1903) (Apidae) *Bombus hortorum* (Linnaeus, 1761) (Apidae) *Bombus hypnorum* (Linnaeus, 1758) (Apidae) LEPIDOPTERA *Amata phegea* (Linnaeus, 1758) (Arctiidae) DIPTERA *Episyrphus balteatus* (De Geer, 1776) (Syrphidae) *Asindulum* sp. (Mycetophilidae)	Jakubska et al. 2005a, 2005c
Epipactis atrorubens (Hoffm.) Besser	HYMENOPTERA: ACULEATA *Bombus* sp. (Apidae) *Vespa soxomica** (Fabricius, 1793) (Vespidae)	Godfery 1933
	DIPTERA *Episyrphus balteatus* (De Geer, 1776) (Syrphidae) HYMENOPTERA: ACULEATA *Bombus lucorum* (Linnaeus, 1758) (Apidae) *Bombus hortorum* (Linnaeus, 1761) (Apidae) *Myrmica rubra* (Linnaeus, 1758) (worker) (Formicidae) *Myrmica schencki* Viereck, 1903 (worker) (Formicidae)	Jakubska-Busse and Kadej current observation
Epipactis palustris (L.) Crantz	Flies, honeybees	Pijl and Dodson 1966
	Diggerwasps, ants, honeybees	Brantjes 1981
	DIPTERA *Sacrophaga carnaria* (Linnaeus 1758) (Sacrophagidae) *Coelopa frigida* (Fabricius, 1805) (Coelopidae)	Proházka and Velisek 1983
	Sawflies, parasitic *Hymenoptera* hoverflies (Holland), ants, wasps	Nilsson 1978
	Bumblebees, hoverflies, mainly bees (Britain)	Proctor and Yeo 1973
	HYMENOPTERA: ACULEATA *Apis mellifera* Linnaeus, 1758 (Apidae) *Bombus lapidarius* (Linnaeus, 1758) (Apidae) *Bombus lucorum* (Linnaeus, 1758) (Apidae) DIPTERA *Calyptrata* sp. (Muscidae) *Empis* sp. (Empididae) *Episyrphus* sp. (Syrphidae)	Jakubska-Busse and Kadej 2008

TABLE 1. Cont.

Species of *Epipactis*	List of pollinators and concomitant insects	References
	COLEOPTERA *Anomala dubia* (Scopoli, 1763) (Scarabaeidae) *Cantharis pellucida* Fabricius, 1792 (Cantharidae) *Gaurotes virginea* (Linnaeus, 1758) (Cerambycidae) *Oedemera femorata* (Scopoli, 1763) (Oedemeridae) LEPIDOPTERA *Ochlodes sylvanus* (Esper, 1777) (Heperiidae) *Aphantopus hyperantus* (Linnaeus, 1759) (Nymphalidae)	Jakubska-Busse and Kadej current observation
Epipactis purpurata Sm.	HYMENOPTERA: *Vespula austriaca* (Panzer, 1799) (Vespidae) male of cuckoo wasps (Chrysididae) *Vespula vulgaris* (Linnaeus, 1758) (Vespidae) DIPTERA *Episyrphus balteatus* (De Geer, 1776) (Syrphidae) COLEOPTERA *Coccinella septempunctata* Linnaeus, 1758 (Coccinellidae)	ANONIMUS 2010 Jakubska-Busse and Kadej current observation

* Currently classified as *Dolichovespula saxonica*. The underlining denotes species classified by authors as pollinators

Xerothermic habitats of *E. atrorubens* are easily accessible to pollinators; additionally, the pollinators are lured by chemical attractants rich nectar. Only in populations close to the seaside, a lower frequency of insect visits was observed, which might be due to a deterring influence of strong and cool winds blowing from the sea. Additonally, the ramets often grow on exposed beaches, subject to intensive sun activity and thus their nectar evaporates faster than in specimens growing in shaded areas. As a result, the ramets become less attractive to insects relatively sooner.

The most interesting and, at the same time, the most controversial species among the examined ones is *E. purpurata* Sm., which grows with limited access to the light and without surrounding blooming plants, which could be a possible food source for potential pollinators or visitors, such as bumblebees (see Fig. 3F). In fact, bumblebees occur mainly in a close proximity to plants that serve them as a food source, e.g. *Chamaenerion angustifolium* (Onagraceae), *Lythrum salicaria* (Lythraceae), *Vicia* sp. (Fabaceae). In the habitats of *E. purpurata* these species were not present, which could explain low frequency of bumblebee's visit.

The differences in nectar chemical composition and the flowers fragrance in *E. purpurata* (see Fig. 1) are hard to determine. The procedure involving the nets, which were supposed to help in assessing the level of autogamy, indicated that the species produced over 82% of flowers and proved that the plant is autogamic. Although the orchid successfully produces nectar rich in attractants, the potential pollinators rarely occur in its habitat which in the end results in less insects visiting the flowers. Therefore, it is possible that autogamy of this species is connected to its limited access to pollinators. Among the examined populations *E. purpurata* produced only flowering shoots, and no vegetative ramets. The only known pollinators of this species are: a male cuckoo wasp (Hymenoptera, Chrysididae), and a hoverfly, *Episyrphus balteatus*, which has also been recorded visiting it (Anonimus 2010). Another potential pollinator of *E. purpurata* is *Coccinella*, as it was observed to accidentally carry pollen.

Floral morphology vs. chemical attractants

The research procedure involving displaying pictures of orchids, was designed to determine whether different shapes and colours of flowers influence the frequency of insects visits. No such relationship, however, was discovered. According to other sources, red flowers should be attractive to flies, but these were equally often seen on white flowers of *E. palustris* or blue ones of *E. purpurata*. Therefore, it seems that the key role in luring biology belongs to chemical attractants.

It was observed that the insects usually did not return to the marked flowers, which can be explained by the presence of other food sources (i.e. melliferous plants) in close proximity to the examined orchids.

The influence of nectar components on insects' behavior posess another fascinating question. The obtained results partially confirm suggestions of Brodmann et al. (2008) on the significance of such compounds as nonanal, decenal, benzaldehyde in the process of luring the insects, particularly Vespidae. Yet, it seems that there are more compounds in the nectar that could influence insects' behaviour, as not only representatives of *Vespa* or *Apis* genera but also other creatures were observed to visit the plants. Furthermore, it should be remembered that nectar is a complex composition of compounds and their derivatives.

The composition of the scent released by *Epipactis palustris* might be the most crucial in initially attracting pollinators and visitor insects. The compounds that were discovered, such as eugenol and vanillin, are strong aromatic attractants as a substantial number of Diptera representatives, which have a sense of smell, visited flowers of *E. palustris*. Among Diptera, *Episyrphus balteatus* (Diptera: Syrphidae) was found to be a pollinator of *Epipactis thunbergii* (Cingel 2001). Furthermore, it seems that *Episyrphus* is a universal pollinator of all flower plants.

Anomala dubia (Scarabaeidae) and *Gaurotes virginea* (Cerambycidae) and Diptera – *Calyptrata* sp. (Muscidae) beetles might be pollinators of *E. palustris*, as they have a desired body size and were often seen on the flowers in question. Yet, no pollen was seen to stick to the bodies of aforementioned beetles.

Orchids visited and pollinated by insects create perfect living and hunting conditions for spiders and predatory froghoppers, which feed on pollinators. The following Heteroptera: *Liocoris tripustulatus* (Miridae), *Lygus pratensis* (Miridae) and Arachnidae species *Metellina mengli* (Tetragnathidae) and *Philodromus dispar* (Philodromidae) were repeatedly observed on orchids. Interestingly, *Misumena vatia* (Araneae) very often landed on the displayed flower pictures and always headed toward its bottom, as it would do on real flowers.

The procedure that introduced isolating inflorescences in order to prevent the flowers from being visited by insects brought an even more surprising conclusion – namely, 74.9% of all examined Helleborine flowers produced fruit, which proves the domination of autogamy. The percentage of autogamy was the highest in *E. purpurata* 82% and in *E. palustris* 79.4%, as opposed to the lowest in *E. atrorubens* 63.5%. However, the findings could be distorted by the qualities of the studied populations.

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