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MECHANISM OF SPONTANEOUS AUTOGAMY IN THE ALLOGAMOUS LEPIDOPTERAN ORCHID *GYMNADENIA CONOPSEA* (L.) R. BR. (ORCHIDACEAE)

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Studies of plant breeding systems are particularly important when they involve facultative processes, which can be overlooked in natural conditions. This especially applies to species that are considered allogamous, due to their pollination syndrome and floral architecture. We examined the potential level and factors enabling or limiting spontaneous autogamy in three populations of the lepidopteran orchid *Gymnadenia conopsea*. Using a bagging experiment, we noted the stages of the anther thecae and the positions of the pollinaria at the five phenological stages of the flower, as well as the quantity of autogamously set fruits and the number of properly formed seeds. In the studied populations, autogamy represents an accidental character, with a maximum of 3.3% of fruits set spontaneously per analysed sample in a given population, and with seed numbers ranging from 29 to 354. This process is an environmentally dependent co-product of the mechanisms that enable a position appropriate for touching the stigma (bending of the caudicle) and increase male fitness (disintegration of the massulae), preceded by the gradual opening of the anther chambers. Autopollination of *G. conopsea* may occur in the flowers at various flowering stages (excluding the beginning of anthesis) at each position on the inflorescence.

Key words: Anther thecae, bending movement, dehydration, disintegrated pollinium.

INTRODUCTION

The breeding system is a crucial aspect of species biology. It is one of the most important factors shaping the demographic and genetic structure of populations, as well as the evolution of genomes (Hamrick and Godt, 1989; Charlesworth, 2006). The evolution of plant breeding systems is particularly evident at the individual level as the adaptation to changes in environmental conditions, both biotic and abiotic (Kalisz and Vogler, 2003; Charlesworth, 2006). In general, the reproductive success of entomophilous plants is strongly pollinator-limited. Thus, most plants are self-compatible, which allows them to achieve mixed mating (Goodwillie et al., 2005). Simultaneously, a permanent or periodic lack of pollen-vectors can also stimulate a transition to autonomous selfing as reproductive assurance (Darwin, 1877; Holsinger, 1996). The total lack of pollinators may become an important selective agent on floral traits, inducing the transition from allogamous to pronounced autogamous flowers (Macnair et al., 1989; Squirrell et al., 2002). The low frequency of pollinators (even periodically) may initiate facultative autogamy, usually in the final phase of anthesis (Kalisz et al., 1999; Suetsugu, 2013). According to Lloyd and Schoen (1992), autogamy at this period has been commonly called delayed, although in reality, autogamous selfing does not fall into strict classes of floral lifespan and is instead continuously distributed (Goodwillie et al., 2005). Because the possibility of late autogamy is a consequence of the habitually or temporarily low frequency of pollinators and because floral traits related to this selfing type may change over floral life, the appearance and extent of facultative autogamy may vary between populations of a given species, and even between years within the same population, and it may therefore be overlooked (Kalisz et al., 1999).

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The adaptation of flowers to limited pollinators through autogamy is particularly noticeable in the Orchidaceae. Delayed autogamy may occur in several orchid species (reviewed in Catling, 1990). Although this process has not been proven empirically, gynostemium structure and pollination syndrome may indicate the possibility of its occurrence. A very simple type of gynostemium may cause a large number of its morphological variations to lead to facultative autogamy as a minor adaptation to local pollinator availability, especially when derived from a generalised pollination system resulting from Hymenoptera and Diptera visits (Squirrell et al., 2002; Tałałaj and Brzosko, 2008). A more specialised architecture that is adapted to flower pollination by Lepidoptera significantly reduces the probability of autogamy (reviewed in Claessens and Kleynen, 2011). Flower features and pollination syndrome are very often the basis for speculation about the possibility of autogamy for the specified orchid, and such information is used in many fields of plant science. Thus, there is a significant need for research on the breeding system potential of orchids.

In the present study, we examine the potential for spontaneous autogamy in lepidopteran *Gymnadenia conopsea* (L.) R. Br., a species widely regarded as allogamous and which is currently the subject of intensive research (Jersáková et al., 2010; Stark et al. 2011; Trávníček et al., 2012; Meekers and Honnay, 2011; Sletvold and Ågren, 2011; Sletvold et al., 2012). The common view is that this plant is self-compatible but depends on pollinators (Gustafsson, 2000; Huber et al., 2005; Jersáková et al., 2010; Sletvold and Ågren, 2011; Sletvold et al., 2012) and that under pollinator deficiency, selfing is conducted via geitonogamy (Meekers and Honnay, 2011). Meanwhile, during research on the breeding system of orchids in Poland, fruits of *G. conopsea* were found after an experiment on spontaneous autogamy (I. Tałałaj, unpubl. data). Thus, in the present work, we specifically (1) evaluate the potential level of spontaneous autogamy under pollinator limitation, (2) investigate the mechanism of autogamy in this species, and (3) examine whether the autogamous fruits contain properly formed seeds. Based on the allogamous architecture of the flowers and the pollination syndrome of *G. conopsea*, we hypothesise that autogamy, if it occurs, is possible at the end of flowering.

MATERIAL AND METHODS

STUDY SPECIES

Gymnadenia conopsea (L.) R. Br. is a perennial species occurring on calcareous soils in grazed meadows and in the margins of marshes and fens, both in well-lit open habitats and in significantly shaded sites (Vakhrameeva et al., 2008; Sletvold et al., 2012). The species is distributed over all of Europe and a considerable part of Asia (Hultén and Fries, 1986). Different cytotypes of *G. conopsea* have been recognised, and they often form mixed-ploidy populations (Marhold et al., 2005; Jersáková et al., 2010; Stark et al., 2011). The generative shoot forms a fairly dense inflorescence, consisting of 27–70 and as many as 87 flowers (Vakhrameeva et al., 2008; personal observation). Flowers are small, pinkish or reddish lilac, with narrow spurs of 11–18 mm long, containing a large amount of nectar (Stpiczyńska and Matusiewicz, 2001; Bell et al., 2009, Jersáková et al., 2010). During both day and night, flowers emit a weak vanilla-like but slightly unpleasant scent (Marhold et al., 2005), which is produced in osmophores on the surface of the labellum and lateral sepals (Stpiczyńska, 2001). Sletvold et al. (2012) present this species as an orchid with a semi-generalised pollination system because it receives visits from a large number of species, though most of them are lepidopteran (listed in Claessens and Kleynen, 2011). In other populations, distinct differences in pollination efficiency between butterflies and moths have been observed (Vöth, 2000; Meyer et al., 2007; Sletvold et al., 2012), as this species may represent an intermediate stage in the adaptation to diurnal versus nocturnal pollinators (Claessens and Kleynen, 2011). The fruiting level varies significantly between different sites and ranges from 23% to 95.5% (compiled in Claessens and Kleynen, 2011).

The gynostemium of *G. conopsea* is very short, massive and deflexed. The anther is joined to the gynostemium above the rostellum, close to the stigma base. The anther contains two parallel chambers covered by thin-walled, attenuated and partially free anther thecae at the base, which open by a slit (Szlachetko and Rutkowski, 2000; Box et al., 2008). Each chamber contains one pollinarium with a clearly distinguishable sectile pollinium (formed by the pollen tetrads, bound together by elastic threads in the massulae) and a long caudicle, which is attached to a sticky viscidium (Darwin, 1877; Freudenstein and Rasmussen, 1997; Claessens and Kleynen, 2011). Caudiculae exhibit the ability of a bending movement when the viscidium adheres to a pollinator's proboscis which brings the pollinia into the appropriate position for touching the stigma (Darwin, 1877). The stigma in *G. conopsea* is located ventral to the anther thecae and consists of three lobes. The two lateral lobes are confluent, each with a flat receptive surface. The basal part of the median lobe is also likely to be fertile, but it is covered with an almost pendant, remnant rostellum (Szlachetko and Rutkowski, 2000; Box et al., 2008).

STUDY SITES

Studies were carried out in 2013, in northeastern Poland, in two natural habitats of the Biebrza Valley (populations ZAB and LIP) and in one habitat in the Rospuda Valley (population ROS). *G. conopsea* is very rare in this region, and the three populations are spaced from c. 30 to 100 km apart from each other. Populations LIP and ROS are located on a transitional mire, and ZAB is situated on the border between a mineral island and a peat bog. In the populations studied, the number of flowering shoots was < 50 in ZAB and ROS and > 50 in LIP. At these sites, *G. conopsea* blooms in June and July and sets fruit in mid-August. These populations are represented only by diploid *G. conopsea* sensu stricto (Trávnícek̆ et al., 2012).

FIELD AND LABORATORY STUDIES

When all the flowers on the inflorescence were in bud, just before blooming, spikes were covered with cotton nets. Based on the size of each population, five inflorescences were covered in ZAB and ROS, and ten inflorescences in LIP. Of all the shoots, one from ZAB and two from ROS were damaged by wild animals. In total, 17 shoots and 558 flowers were included in the analysis. During flowering, each population was monitored at three blooming stages: (1) when most flowers on the inflorescence were freshly opened and the upper flowers were at the bud stage, (2) when flowers on the inflorescence were differentiated in terms of the quality of the sepals, and (3) when all flowers were withered. During each visit, nets were removed, and each flower was carefully inspected (using a 10x magnifying glass) for three traits, which were defined after the preliminary analysis of the flower stages of *G. conopsea*: quality of the sepals: S.1. fresh, S.2. early wilting, S.3. withered, S.4. shrivelled; stage of the anther thecae (Fig. 1a–d), able or unable to move the pollen outside the chamber (analysed separately for the left and right chambers): A.1. initial (partially free at the base), A.2. with a narrow gap, A.3. half-opened, A.4. fully opened; position of the pollinarium (analysed separately for the left and right) (Fig. 1a–d): P.1. compact pollinium inside the chamber, P.2. disintegrated pollinium with massulae inside the chamber, filling it fully and even with massulae protruding from the chamber, P.3. pollinium bending forward on the caudicle, outside the chamber. After detailed analysis of the possible duration of the subsequent quality stages of the sepals, for further statistical analysis, we established the following phenological stages of the flower of *G. conopsea*: PH.1. fresh sepals at the first blooming stage, PH.2. fresh sepals that were fresh at the previous blooming stage (long-fresh), PH.3. the beginning of wilting, PH.4. withered sepals, PH.5. shrivelled sepals.

At fruit maturation, we recorded the number of fruits and their position on the inflorescence. Fruits were collected, and autogamous seeds were counted under a dissecting microscope. Seeds of *G. conopsea* are dust-like, weighing approximately 2–8 μg and measuring 0.62 (±0.1) mm in length, but the embryo is clearly visible through a transparent testa (Rasmussen, 1995; Arditti and Ghani, 2000). During laboratory analysis, only properly formed seeds, i.e., those with clearly visible and swollen yellow embryos, were considered. Seeds with abnormally shaped embryos, or those differing in colour, were rejected as aborted (Goodwillie and Knight, 2006). As a reference, the seed numbers from the fruit sets under natural pollination were counted. Fruits were collected from randomly chosen shoots that were available from the ZAB and LIP populations.

DATA ANALYSIS

Significant differences in anther opening percentages at particular flowering stages (also calculated separately for the three positions on the inflorescence) were determined by a multiple comparison test for proportions (Zar, 1996). When differences among groups were found, paired comparisons were performed. For the reference values below 5, Yates's correction was applied. During these analyses, records from the middle flowers at the last phenological stage (PH.5.) were not included because a high share of mouldy chambers could significantly distort the result. To compare the degree of opening of the anther chambers at the end of anthesis, the chi-square test was used. To judge the overall statistical significance of a series of chi-square analyses obtained for individuals representing the same category of the analysed parameter, Jost's (2008) formula was applied:

$$P = k\sum_{i=0}^{n-1}\frac{(-\ln(k))^{i}}{i!}$$

where P is the combined significance level from multiple analyses and k is the product of the given set of p-values. Analyses were performed in IBM SPSS Statistics software, ver. 21.

RESULTS

ANTHER OPENING

At the beginning of anthesis (PH.1.), we noticed a significantly higher frequency of anther cells at the initial stage (A.1.) compared to other anther stages (Fig. 2a, Tab. 1). Chambers were also recorded with a narrow gap (A.2.) with a significantly higher frequency (paired comparisons chi-square, $P < 0.001$)

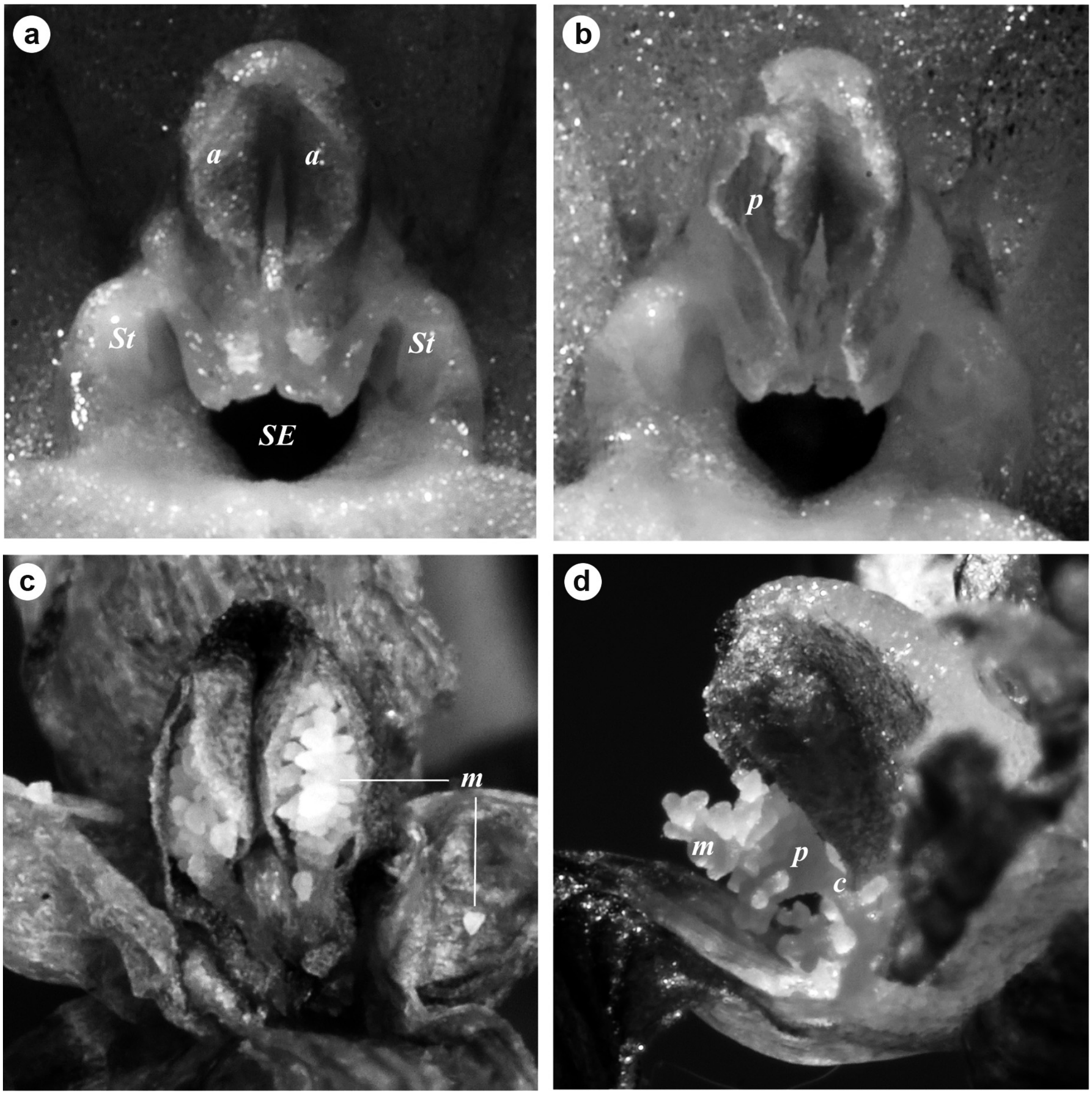

Fig. 1. View of the column of *Gymnadenia conopsea* at different stages of the anther thecae and different positions of the pollinaria. (**a**) Chambers at initial stage, (**b**) Left chamber half-opened with a compact pollinium inside, right chamber with a small narrow gap at the bottom, (**c**) Fully opened chambers with disintegrated massulae inside and outside the chambers, (**d**) Right pollinium disintegrated and leaning forward, side view. a – anther cell; St – lateral lobe of the stigma; SE – Spur Entrance; p – pollinium; m – massula; c – caudicle.

than either the rarely seen half-opened chambers (A.3.) or the least frequently observed fully opened chambers (A.4.). At the long-fresh stage (PH.2.) and at the beginning of wilting (PH.3.), chambers A.3. and A.4. gradually increased in frequency, and did so at similar levels, (paired comparison chi square, $P > 0.05$), but they were still significantly less common than the most dominant anther cells at the A.1. stage (paired comparisons chi square, $P < 0.001$) and the intermediate stage A.2. (paired comparisons chi square, $P < 0.05$). At the withered stage of the flower (PH.4.) the frequencies of the anther chambers at particular stages were equalled at a similar level. This situation persisted until the last stage of the flower (PH.5.), in which only half-opened chambers were significantly less frequent than chambers at A.1. and A.4. stages (paired comparisons chi square, $P < 0.01$) (Fig. 2a, Tab. 1). The degree of the opening of the anther chambers differed in some respects between the flowers at three positions on the inflorescence (Fig. 2b–d, Tab. 1). Among particular phenological stages, flowers at the bottom presented the highest frequency of fully opened anther cells (A.4.) at the end of flowering (PH.5.) (Fig. 2b, Tab. 1). In the upper flowers, half-opened chambers (A.3.) were almost absent until stage PH.5. (Fig. 2d).

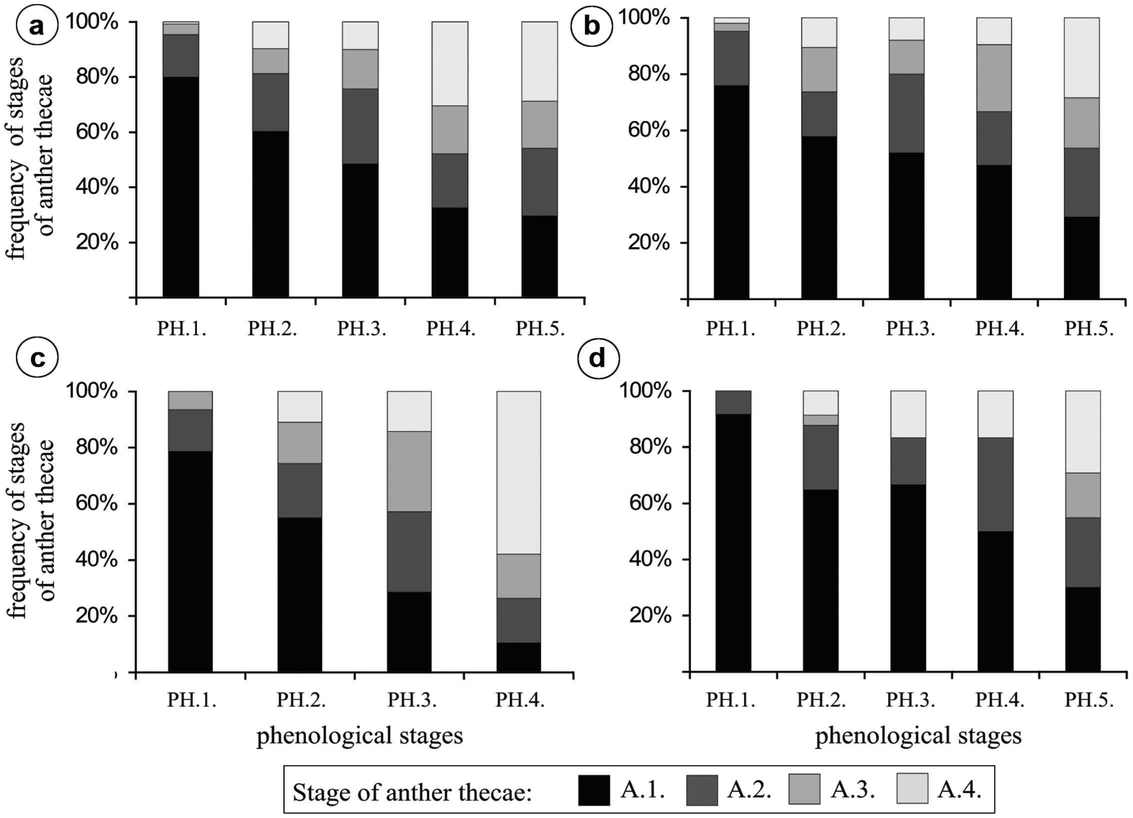

Fig. 2. Frequency of the four anther stages at the five phenological stages of the flower. (**a**) All the inflorescence, (**b**) Bottom flowers, (**c**) Middle flowers, (**d**) Upper flowers. Abbreviations are explained in Materials and Methods.

TABLE 1. Multiple comparison test for proportions of the four stages of the anther thecae at the five phenological stages of the flower.

Phenological stage	All inflorescence			Bottom flowers			Middle flowers			Upper flowers		
	x^2	df	*P*	x^2	df	*P*	x^2	df	*P*	x^2	df	*P*
PH.1.	431.81	3	< 0.001	151.92	3	< 0.001	101.17	2	< 0.001	33.33	1	< 0.001
PH.2.	187.07	3	< 0.001	11.10	3	0.011	53	3	< 0.001	128.42	3	< 0.001
PH.3.	25.2	3	< 0.001	23.92	3	< 0.001	0.86	3	0.836	3	2	0.223
PH.4.	3.22	3	0.359	6.62	3	0.085	11.11	3	0.011	1	2	0.607
PH.5.	19.30	3	0.023	3.99	3	0.262	-	-	-	5.69	3	0.128

Significant results ($P < 0.05$) are shown in bold. Abbreviations for the phenological stages are explained in Materials and Methods.

At this position, the first fully opened anther cells (A.4.) were recorded at PH.2., and their frequency increased at PH.3. and again at PH.5. The highest discrepancies were observed in the middle flowers (Fig. 2c, Tab. 1). At this position, from stage PH.1. to stage PH.4., we noticed a strong decline in the frequency of chambers at initial stage (A.1.), and at stage PH.4., there was a significantly higher frequency of fully opened chambers (A.4.) (paired comparisons chi-square, $P < 0.05$).

The frequency of opening of the anther chambers observed at the end of the flowering period (PH.5.) differed between particular individuals (without significant differences between the studied populations), and they were organised into three subsets:

- in 10 individuals, A.1. and/or A.2. = A.3. and/or A.4. ($P < 0.001$),
- in 5 individuals, A.1. and/or A.2. > A.3. and/or A.4. ($P < 0.001$),

- in 2 individuals, A.1. and/or A.2. < A.3. and/or A.4. ($P < 0.001$).

For the majority of the inflorescences (13), if flowers begin to open the chambers more widely (stages A.3. and A.4.), 60 to 100% of the flowers on the spike open both anther caps. For four individuals, we noticed that more often (67–100%), one chamber within the flower was half or fully opened at the end of anthesis.

POLLINARIUM PRESENTATION

Among the 558 flowers included in the analysis, 28 showed at least one pollinarium at stage P.2. (disintegrated pollinium inside the chamber) or P.3. (pollinarium outside the chamber). This observation was recorded for nine individuals: three individuals from LIP, four from ZAB and two from ROS. Most inflorescences included one or two flowers with a pollinarium at P.2. or P.3. Only for one inflorescence did we notice four flowers (14% of all flowers per shoot), and for another inflorescence 15 flowers (43% of all flowers per shoot), with at least one pollinium no longer compact. Among the 28 flowers with at least one disintegrated pollinium (P.2.) or outside the chamber (P.3.), four flowers from three individuals were located at the bottom of the inflorescence, 10 flowers from four individuals were at the middle part of the inflorescence, and 14 flowers from six individuals were at the upper position of the inflorescence. Among the flowers at the bottom, only when sepals were shrivelled (PH.5.) did we notice half of the pollinaria at the P.2. stage and half at the P.3. stage. In the middle, we detected that most of the pollinaria were disintegrated when sepals were long fresh (PH.2.) or when sepals were shrivelled (PH.5.). At this position on the inflorescence, a single event of pollinaria bending was detected during the beginning of wilting (PH.3.), and another was observed when sepals were withered (PH.4.). Among the flowers at the upper position on the inflorescence, half of the pollinaria were at stage P.2., and half were at stage P.3. However, at this position, disintegrated pollinia inside the chamber were detected at three phenological stages of the flower: PH.2., PH.4. and PH.5. Pollinaria outside the chamber were recorded at two phenological stages of the flower: PH.2. and PH.5., which included the largest share (Fig. 3).

At the end of anthesis, of all the half-opened chambers (A.3.), 99% contained a compact pollinium inside the chamber (P.1.), and only 1% included a disintegrated pollinium (P.2.). In contrast, of all the chambers fully opened (A.4.) at the end of anthesis, 74% included a compact pollinium (P.1.), 19% a disintegrated pollinium (P.2.) and 7% included a pollinium bent forward on the caudicle, outside the chamber (P.3.).

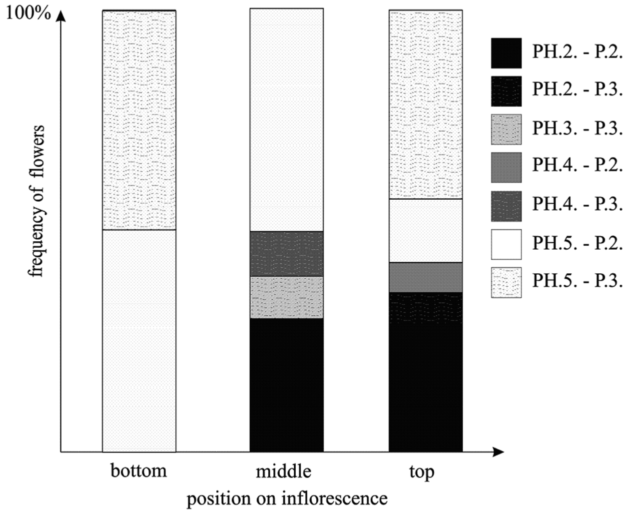

Fig. 3. Frequency of the flowers with at least one pollinium disintegrated inside the chamber P.2. (boxes with pure colour) or pollinium bent outside the chamber P.3. (boxes with texture) at the different phenological stages of the flower, depending on the position on inflorescence. Abbreviations are explained in Materials and Methods.

REPRODUCTIVE SUCCESS

After the bagging experiment, only four fruits on two individuals in the LIP population, two fruits on two individuals in the ZAB population, and two fruits on one individual in the ROS population were observed (Tab. 2). Two fruits were set at the bottom part of the inflorescence, one in the middle, and five at the upper position. Flowers that set fruits autonomously had one or two fully opened chambers (A.4.). Among the recorded fully opened chambers (not damaged), pollinaria were observed three times at position P.3. and once at position P.2. Each of the autogamous fruits contained properly formed seeds. Their numbers ranged from 29 to 354, without any correlation with the fruit position on the inflorescence (Tab. 2). For the randomly chosen, naturally set fruits (four fruits each from the ZAB and LIP populations), the number of properly formed seeds varied from 628 to 2417 (Tab. 3). Seeds number was significantly decreased towards the top (Spearman's rank correlation, $r = -0.95$, $P < 0.005$; open capsules were excluded).

DISCUSSION

REPRODUCTIVE SUCCESS AFTER AUTOGAMY

The results of this study reject the model that spontaneous autogamy is impossible in the lepidopteran orchid *Gymnadenia conopsea* (Gustafsson, 2000; Huber et al., 2005; Sletvold and Ågren, 2011).

TABLE 2. Properties of auto-pollinating flowers at the final phase of anthesis and the number of properly formed autogamous seeds. n – flower position on the inflorescence (counted from the bottom up), N – the total number of flowers on the inflorescence, NA – data not available due to flower (the column) or fruit damage.

Population	Individual	Position on inflorescence [n/N]	Left chamber	Right chamber	Left pollinarium	Right pollinarium	No. of seeds
LIP	3	21/28	narrow gap	fully opened	pollinium compact, inside the chamber	caudicle bent, pollinium outside the chamber	65
LIP	6	12/55	narrow gap	fully opened	pollinium compact, inside the chamber	disintegrated pollinium, massulae inside/outside the chamber	44
LIP	6	28/55	NA	NA	NA	NA	154
LIP	6	37/55	fully opened	initial stage	caudicle bent, pollinium outside the chamber	pollinium compact, inside the chamber	354
ZAB	1	9/34	NA	NA	NA	NA	NA
ZAB	2	25/35	fully opened	fully opened	NA	NA	37
ROS	3	22/26	fully opened	fully opened	pollinium compact, inside the chamber	caudicle bent, pollinium outside the chamber	205
ROS	3	24/26	NA	NA	NA	NA	29

Although we based this on a very limited number of sampled plants, in each of the studied populations we noticed very low levels of autogamously set fruits (varying from 0.2% to 3.3% per analysed sample in a given population), which confirms the rare events of spontaneous autogamy in populations from England (Campbell, 1999) and Czech Republic (Jersáková and Kindlmann, 2004), and does not exclude the potential of higher rates of autogamy, which was recorded at 36% fruiting level after complete plant exclusion from pollinators in a population from Germany (Meyer et al., 2007). Independent of the position on inflorescence, fruits set autogamously contain a significantly lower number of properly formed seeds than fruits on control plants (regardless of whether they were derived from crossing, geitonogamy or even facilitated autogamy), an observation that was also reported by Meyer et al. (2007). In *G. conopsea*, eleven critical stages in the development of the ovule and seed have been revealed (Shamrov and Anisimova, 2003), which significantly increases the number of stages during which pre-zygotic selection may act. Jersáková et al. (2010) and Huber et al. (2005) recorded a significant inbreeding depression in artificially selfed seeds of this species. Certainly, the huge discrepancies in the numbers of autogamously set fruits also result from the number of massulae that reach the stigma (Neiland and Wilcock, 1995), through a mechanism that we describe below.

TABLE 3. The number of properly formed seeds per fruit set after natural pollination.

Population	Individual	Position on inflorescence	No. of seeds
ZAB	1	bottom	2417
	2	bottom	2099
	3	bottom	≥870
	4	bottom	≥1040
LIP	1	top	790
	2	top	1101
	3	middle	1205
	4	top	≥628

≥ – seed number equal or greater because fruit was slightly open.

THE MECHANISM OF ANTHER OPENING

Based on our observations, the first step enabling self-pollination in *G. conopsea* is a wide-open chamber whose pollinia disintegrates or bends forward. This occurs most often when chambers are open. In the Orchidaceae, total or partial opening of the anthers depends on different patterns of thickening of the entire secondary wall, which is part of an individual endothecial cell (Freudenstein, 1991; Keijzer, 1999). The thickening is usually not uniform over the cell walls but is instead composed of bars that surround the protoplast, and individual thickening in orchids may be composed of one to many helical or annular bars. This thickening may be open, half-open, or closed. *G. conopsea* shows type II thickening (also restricted to other members of Orchideae), which includes widely spaced rings and no connection between thickenings (Freudenstein, 1991). Wall thickenings are responsible for the change of volume and cell shape during dehydration, causing stretching and folding. This leads to the opening of the anther and pollen presentation (Bianchini and Pacini, 1996, Peter and Johnson, 2014). When the pollen dispersal unit (PDU) is a pollinium, as in Orchideae, the anther cap flaps may be half-opened at most (Pacini and Hesse, 2004). Thus, half-opening means that pollinaria are partially enclosed by the anther, unlike the pollen, which is exposed in the fully opened anthers in monads and tetrads (Pacini and Hesse, 2004), and this half-opening corresponds to the wide-open stage in the present study.

In the frequently autogamous orchids *Pseudorchis albida*, *Neotinea maculata*, *Gennaria diphylla* Parl. and *Serapias parviflora* Parl. belonging to the same Orchideae tribe as *G. conopsea*, the anther cells open widely just before or at the beginning of anthesis (Moggridge, 1865; Claessens and Kleynen, 2011). In *G. conopsea* we noticed a gradual opening of the anther thecae until the beginning of wilting, and there was an equalisation of the frequency of initial covering and wide-open chambers at the withered stage of the flower. Although the intensity of anther flaps opening is similar within the same flower (therefore, if a flower begins to open a chamber, both chambers will open) it is very irregular among individuals and even within the same inflorescence. The primary mechanism behind the gradual, delayed opening of the anther cap is the changing water status of its cells (Catling and Catling, 1991) and water loss is strictly dependent on environmental conditions such as rain, humidity and sunlight, as well as variations in relative humidity (RH) with the time of day (Catling and Catling, 1991; Yates and Sparks, 1993; Lisci et al., 1994; Bianchini and Pacini, 1996; Peter and Johnson, 2014).

CONDITIONS OF THE POLLINARIA PRESENTATION THAT PROMOTE AUTOGAMY

The majority of the pollinia inside the open anther cells of *G. conopsea* are compact. We observed that only a quarter of the pollinia inside the open chambers were disintegrated or bent forward. The PDU of *G. conopsea* belongs to the type most common in the family, which is a soft pollinium type C, in which the elastoviscin threads that hold the massulae together are not very strong (gathered in Pacini, 2009), and a slight loss of water may cause the detachment of some massulae from the caudicle and a partial breakup of the pollinium, while pollen grains, especially those inside the massula, may be compressed (Pacini and Hesse, 2004; Pacini, 2009). Rarely, pollinia dehydration causes shrinking of the whole pollinium as in *Bulbophyllum involutum* (Borba and Semir, 1999).

In orchids, if the pollinium is protected by the anther wall, it may lose some water, determined by the relative humidity of the air during the environmental presentation of the pollinium (Pacini and Hesse, 2002). Environmental presentation occurs during the export by the pollinators, when pollinaria lean forward from the chamber (we noticed that each pollinium outside the chamber was disintegrated), and it may occur when anther cells are open. Our results indicate that in *G. conopsea*, efficiency of water loss during pollen presentation inside the anther (corresponding with disintegrated pollinium) is very low, and it depends on the degree of anther cell opening (disintegrated massulae were observed in 19% of fully opened chambers and only in 1% of half-opened chambers) but does not depend on the phenological stage of the flower (pollinia became disintegrated with a similar frequency in long-fresh and withered flowers). In autogamous members of Orchideae, in which disintegrated massulae play an important role in auto pollination, water loss from the pollinium presented in a wide-open chamber is very fast and highly efficient at the beginning of anthesis (Claessens and Kleynen, 2011). In such species, there are also additional facilities to deposit pollen fragments that have fallen out from the open chamber onto the stigma. In autogamous *S. parviflora*, the flower stands upright, such that massulae fall directly onto the stigma (Moggridge, 1865). In *P. albida*, all of the flowers lean over, and in *N. maculata*, both lateral lobes of the stigma protrude (Claessens and Kleynen, 2011). In *G. conopsea*, the column is erect, and although the stigma is beneath it, two spacious, receptive female surfaces are located laterally (Box et al., 2008). It is evident that this positioning of male and female structures reduces the probability of pollen falling onto the stigma when disintegrated pollinia are inside the chamber in comparison to the disintegrated pollinia being outside the anther cell. Although in *G. conopsea*, caudicle-bending movements were recorded at a significantly lower level (7%) than disintegrated pollinia inside the chambers (20%), more autogamous fruits were set in the flowers with pollinaria leaning forward. Therefore, disintegration of the pollinia presented outside the chamber and the setting of massulae close to the stigma surface play important roles in the self-pollination of *G. conopsea*.

The bending movement of the caudicles on the body of the pollinator affects most European orchids. The bending time is an important factor that may prevent deposition of the pollen onto the stigma of a flower in the same inflorescence (Darwin, 1877; Johnson and Edwards, 2000). This movement is a hygroscopic process and is caused by the rapid dehydration of the tissue where the caudicle is connected to the viscidium (Darwin, 1877). In autogamous *S. parviflora*, *P. albida*, *G. diphylla* or *Ophrys apifera* Huds., in which anther dehydration is very fast before or at the beginning of anthesis, the caudicle facultatively or obligatorily leans forward at this stage (Darwin, 1877; Claessens and Kleynen, 2011). We suggest that in *G. conopsea*, bending within the flower is associated with a rapid decrease in the water balance of the plant. In this species, flowers on the inflorescence open sequentially from the lowest flower up and if the flowers have not been pollinated, they stay fresh for several days (Vallius et al., 2006; Sletvold et al., 2012; personal observation) as an adaptation to maximise the opportunity for pollination (Catling and Catling, 1991). During the experimental exclusion from pollinators, all the flowers on the inflorescence were open, which severely decreased the water status of the plant and influenced the sequential wilting of the flowers from the lowest to the uppermost. Under these circumstances, we observed two moments at which *G. conopsea* suffers the most severe water loss. The first occurred when the bottom flowers were shrivelled, the middle flowers were between the beginning stages of wilting and being fully withered, and the upper flowers were between the long-fresh stage and starting to wilt. At these positions and phenological stages, we noticed an increase in the wide opening of the chambers in comparison with the previous stage (extremely common in the middle flowers), and we recorded the bending movement of the caudicles only in those flowering stages and positions. We observed the second time of intensive plant dehydration at the end of wilting of all the inflorescences, when the upper flowers with shrivelled sepals possessed pollinaria that bent forward.

After self-pollination, the duration of male and female receptivity plays a crucial role in self-fertilisation. Neiland and Wilcock (1995) reported that in *Orchis morio* L., self-pollinated flowers were able to produce at least some fruits during the entire period of pollen germinability, lasting 20 days, although there was a decline in the percentage of fruit formation with time. Similarly, even in *G. conopsea*, pollen remains germinable for at most 37 days after anthesis (Neiland and Wilcock, 1995), and the probability of self-fertilisation decreases in the subsequent phenological stages of the flower. This may be the reason for the higher numbers of fruit sets on the upper flowers, in which pollinia were bent in long-fresh flowers.

CONCLUSIONS

We conclude that in *G. conopsea*, autopollination may occur on the flowers at each position of the inflorescence and at various flowering stages (excluding the beginning of anthesis). This result rejects our prediction that autogamy is only delayed, and it confirms the model in which the timing of autonomous self-pollination is continuously distributed and can be related to floral age or developmental stage (Kalisz and Vogler, 2003), as well reflecting plasticity in floral longevity (Arathi et al., 2002).

It is evident that *G. conopsea* does not possess a specialised adaptation for self-pollination and spontaneous autogamy is an accidental process, a co-product of mechanisms that are directly related to the features of the pollinaria that enable appropriate positioning for touching the stigma (bending of the caudicle) and increase male fitness (massulae disintegration that allows the deposition of one pollinium on many stigmas). Both mechanisms are most common in the representatives of the Orchideae tribe in which autogamy has been reported. Of these species, 50% perform only accidental autogamy, whereas in 30%, autogamous pollination is questionable (gathered in Claessens and Kleynen, 2011). Orchids without specialised mechanisms of self-pollination mainly show frequencies of autogamous events that are unclear, very low or differentiated between sites or years because autogamy is dependent on the local biotic and abiotic conditions. In naturally open plants the spontaneous autogamy will be significantly influenced by the pollinator availability and thus availability of unremoved pollinia. Abiotic factors can influence the degree of self-pollination expressed even by individual plants in the population (Kalisz and Vogler, 2003). Therefore, different populations of *G. conopsea* show discrepancies in spontaneously set fruits (Campbell, 1999; Gustafsson, 2000; Jersáková and Kindlmann, 2004; Huber et al., 2005; Meyer et al., 2007; Sletvold and Ågren, 2011; present study).

AUTHORS' CONTRIBUTIONS

IT devised the concept of the study, carried out the experiments, analysed the data, elaborated the results, prepared the figures and tables and wrote the paper; MS analysed the data and prepared the figure and table. The authors declare that there are no conflicts of interest.

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