Does pollination syndrome reflect pollinator efficiency in *Silene nutans*?

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ABSTRACT

Despite the specialization of floral traits towards particular floral visitors (pollination syndrome), insect-pollinated plants can display generalist rather than specialist interactions with their pollinators. This is the case of several *Silene* species that were viewed as nocturnally pollinated but are factually visited, and then potentially pollinated, by both nocturnal and diurnal insects. This opens the question of the consistency of pollination syndromes and the impact of different pollinator assemblages on plant reproductive success. In this work, we measured diurnal and nocturnal pollinators' contributions to pollination success (fruit and seed production) and offspring quality (seed mass and germination rate) of hermaphrodite plants in an artificial population of *Silene nutans,* by experimentally restricting pollinator access and controlling for floral and resource traits. We found that fruit and seed production was higher for nocturnal pollination, and that only very few seeds were produced by diurnal visitors. In our experimental system, plants did not benefit from being open during daytime, as they showed no fruit or seed production advantage in the open pollination treatment compared to the nocturnal one. Seeds generated from diurnal pollination were heavier but did not germinate better, so that the plant fitness proxy remained higher for nocturnal pollination than diurnal one, without any obvious evidence of a pollination mixed strategy. These data are in accordance with the nocturnal pollination syndrome of *S. nutans*, contrasting with several other studies carried out on other *Silene* species.

1. Introduction

Angiosperms are the most diversified group of vascular plants with more than 250,000 described species (Soltis and Soltis, 2004). The congruent rise of flowering plants and numerous phytophagous insects, including pollinators that have specialized on pollen, has led to the notion of co-radiation between these lineages (Soltis et al., 2005). Actually, the majority of angiosperms rely on pollinating insects for their sexual reproduction (Kearns et al., 1998; Ollerton et al., 2011; Albrecht et al., 2012). Because floral visitors of a given plant species potentially vary in their behavior, their morphology and their local occurrence, they usually also vary in their contribution to plant pollination (Faheem et al., 2004; Young et al., 2007; Ortega-Baes et al., 2011; Holmquist et al., 2012). Therefore, insect-plant associations lead to flowers with specific combinations of traits correlated with particular floral visitors (Bronstein et al., 2006), which maximize the interaction with the most efficient pollinators (*i.e.* "most effective pollinator principle" developed by Grant and Grant, 1965 and by Stebbins, 1970) and minimize pollen loss by narrowing the spectrum of pollen-feeding visitors (Westerkamp, 1996; Westerkamp and Claben-Bockhoff, 2007). The access of such specialized flowers is limited to a guild of specialized and faithful pollinators (Westerkamp, 1997), which increases opportunities for the evolution of specialization and subsequent diversification in angiosperm flowers (Vamosi and Vamosi, 2010). Plant and flower size, color and constriction of the corolla, presence of a landing platform, quantity and quality of nectar, scent, timing of flowering as well as the relative positions of the male and female reproductive organs are the consequences of selective pressures imposed by particular pollinators attracted by floral rewards (Darwin, 1862; Faegri and van der Pijl, 1979; Fenster et al., 2004). These suites of adaptive characters associated with specific groups of pollinators are described as the "pollination syndromes" (Baker and Hurd, 1968; Wyatt, 1983; Fenster et al., 2004).

The validity of this pollination syndrome concept is nevertheless more and more challenged since insect-pollinated plants may be visited by many species, displaying generalist rather than specialist interactions with their pollinators (Pellmyr and Thompson, 1996; Waser et al.,

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1996; Johnson and Steiner, 2000; Fenster et al., 2004). Actually plant-pollinator associations are highly diversified, and specialization in pollination systems is better reflected by the reality of a continuum rather than by a simple dichotomy between specialization and generalization (Waser et al., 1996). Life history, abundance and breeding systems of plants are as many factors that influence the evolution of floral specialization (reviewed in Johnson and Steiner, 2000). The species-rich tropical and temperate floras from the Southern hemisphere are known to display highly specialized pollination systems, often relying on a single pollinator species (Faegri and van der Pijl, 1979; Nilsson et al., 1985; Steiner and Whitehead, 1990; Cox et al., 1991; Armbruster, 1993; Renner and Feil, 1993; Johnson and Bond, 1994; Johnson, 1996; Johnson and Steiner, 1997; Manning and Goldblatt, 1997; Momose et al., 1998). By contrast, the floras in most of Europe and in the eastern and northern parts of North America are dominated by more generalized pollination systems, even for plant species displaying specialized floral traits (Herrera, 1996; Ollerton, 1996; Waser et al., 1996). For instance, many plant species have evolved special features for nocturnal or diurnal pollination such as composition and timing of flower scent emission, floral display and phenology, and dynamics of nectar production (Witt et al., 1999; Jürgens et al., 2002; Ortega-Baes et al., 2011), but they also exploit co-pollinators active in the alternative period (Macgregor et al., 2017).

Regarding nocturnal *versus* diurnal pollination, the *Silene* genus (Caryophyllaceae) is a particularly valuable model to study the adequacy of pollination syndrome for several reasons. First, many *Silene* species have been *a priori* classified as either nocturnal or diurnal, mainly based on their flower color (white flowers being assigned to nocturnal pollination, other species often exhibiting pink flowers being considered as day-pollinated), but recent studies have revealed that the pattern of scent emission often mismatch with this *a priori* syndrome (Prieto-Benitez et al., 2015). Second, several species, viewed as nocturnally pollinated, have been shown to be visited by both nocturnal (mainly moths from Noctuidae, Geometridae and Sphingidae families) and diurnal insects (flies from the Syrphidae family and bees from the Apidae family) (Jürgens et al., 1996, 2002; Jürgens, 2004; Kephart et al., 2006; Reynolds et al., 2009; Buide et al., 2015). Third, among the nocturnal pollinators of *Silene* species, the moths of the genera *Hadena* (Noctuidae) and *Perizoma* (Geometridae) also use the host plant tissues (*i.e.* flowers and developing fruits) as a "nursery" for the growth and development of their larval offspring, sometimes leading to a high fitness cost for the plant (Pettersson, 1991a; Westerbergh, 2004; Kephart et al., 2006). The outcome (mutualistic vs. antagonistic) of such interaction depends on the relative density and efficiency of nursery pollinators and co-pollinators (Giménez-Benavides et al., 2007; Reynolds et al., 2012). For example, whereas the interaction between *Silene stellata* and *Hadena ectypa* seems overall parasitic, it may tend to mutualism when the nursery pollinator occurs at high density and early in flowering (Reynolds et al., 2012). Besides this importance of the relative density of nocturnal vs. diurnal visitors, one important point is their difference in behavior during flower visitation: while moths visit fewer flowers per plant and travel further between plants (Linhart and Mendenhall, 1977; Barthelmess et al., 2006), flies move relatively short distance between plants (Schmitt, 1983; Olesen and Warncke, 1989; Widén and Widén, 1990) and bumblebees (Apidae) perform multiple visits to flowers on a single plant (from the bottom to the top of the inflorescence) or to near neighbors (Pyke, 1978). Such difference in foraging behavior can affect pollination quality. Indeed, long inter-flower flight distances (moths) are likely to result in high rates of cross-pollination (Schmitt, 1980; Herrera, 1987) whereas short inter-flower flights (flies and bumblebees) may promote inbreeding, either through self-pollination (Herrera, 1987) or through crosses between relatives (Barthelmess et al., 2006). In case of inbreeding depression, such restricted pollen flow should thus reduce the overall benefit of diurnal co-pollinators.

All of this opens the question of the consistency of pollination syndromes and the impact of different pollinator assemblages on plant reproductive success. The effectiveness of flower specialization in *Silene* genus is currently questioned. For several species, floral visitors, that are not predicted as pollinators based on the observed pollination syndrome (e.g. diurnal pollinators in white flowers species), are either more or equally efficient pollinators than expected ones (Giménez-Benavides et al., 2007; Reynolds et al., 2012; Prieto-Benitez et al., 2016) or at least contribute to a large part of seed production (Scopece et al., 2018). Many *Silene* species remain however unexplored and their strict dependency on their "expected most efficient" pollinators is largely unknown. In this study, we investigated the consistency of the moth pollination syndrome in *Silene nutans*. This species releases volatile compounds during night (Jürgens et al., 2002), and has been described as moth-pollinated, although active foraging by diurnal insects has also been reported (Hepper, 1956; Jürgens et al., 2002). Specifically, we set out to determine the relative contribution of nocturnal versus diurnal pollinators to the reproductive success of *S. nutans*, taking floral and resource traits (*i.e.* number of flowers, plant height, daytime and nighttime corolla diameter, corolla depth, calyx length and width, petal length and width, pollen production and viability, nectar production and quality) into account, as they are important components for attracting pollinators (Reynolds et al., 2009). For this purpose, we induced diurnal and nocturnal pollination regimes with non-overlapping day-night periods as well as an open pollination regime in an experimental plant population. Based on the nocturnal pollination syndrome classically attributed to *S. nutans*, we hypothesized that (i) pollination efficiency and so plant fitness in terms of fruit production, seed production and seed quality (*i.e.* seed mass and germination rate) is higher in the nocturnal pollination regime compared to the diurnal one, but that (ii) plants benefit from being open in the daytime.

2. Material and methods

2.1. Study species

Silene nutans (Caryophyllaceae) is an herbaceous, often glandular-hairy, long-lived rosette-forming perennial plant species, usually occurring in dry, open grass communities on rock outcrops. Its continental distribution extends from western and northern Europe to central Siberia and the Southern Caucasus (Hepper, 1956; Fitter, 1978). It has been described as a gynomonoecious-gynodioecious species with female, gynomonoecious (*i.e.* plants bearing both perfect and pistillate flowers) and hermaphrodite individuals found in natural populations (Jürgens et al., 2002; Dufay et al., 2010; Garraud et al., 2011). The flowering period extends from mid-April to mid-July (De Bilde, 1973; Hauser and Weidema, 2000). The inflorescence is a lax, often nodding panicle. The flowers are usually creamy-white to greenish and pinkish, with deeply bifid petals. Flowers are protandrous and open at dusk. The first whorl of five anthers dehisces on the first night after opening (stage F1), followed by the second whorl during the second night after opening (stage F2). Stigmas become receptive on the third evening (stage F3), and remain receptive for about two additional days (stages F4-F5) (Fig. 1; Hepper, 1956). During the day, the flowers can be inconspicuous because the petals are rolled back. Each night the petals unfurl and the flowers emit an intense suite of volatile compounds that are commonly found in moth-pollinated flowers, such as benzyl acetate (benzenoid ester), *trans*-β-ocimene (monoterpene) and small amounts of nitrogen-containing compounds (Knudsen and Tollsten, 1993; Jürgens et al., 2002). Moreover, as plant-pollinator interactions entail a matching between proboscis length and corolla depth (Stang et al., 2006; Johnson et al., 2017), the long calyx tube of *S. nutans* is expected to lead to ecologically specialized interaction with longer-proboscid moth species while excluding shorter-proboscid ones. However, calyx length of flowers also matches with bee and fly proboscis lengths, which suggests that pollination may rely on a more diverse group of pollinators (Jürgens et al., 2002). In addition to nocturnal and crepuscular
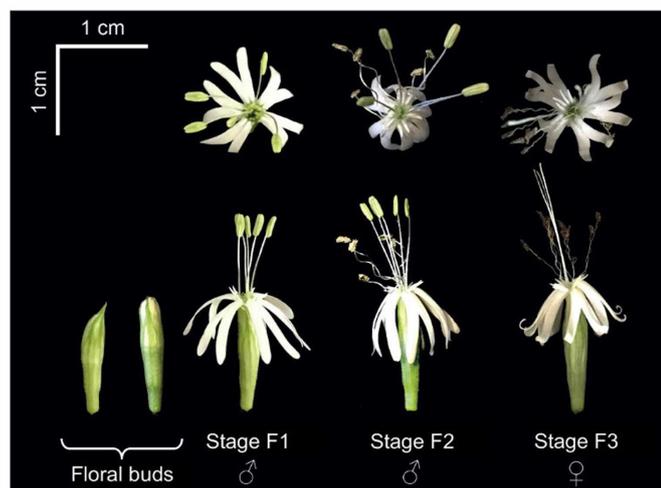
Fig. 1. Floral characteristics and phenology of the male and female functions in hermaphrodite flowers of *Silene nutans*. **Stage F1:** male phase, first night of anthesis. **Stage F2:** male phase, second night of anthesis. **Stage F3:** female phase, third night of flower development.

insects (mainly Noctuidae, Geometridae and Sphingidae) including specialist nursery pollinators (mainly *Hadena* and *Perizoma* genera), flowers of *S. nutans* are indeed regularly visited by diurnal insects, including long-tongued bees (*i.e.* pollen-collecting bees, nectar-collecting bumblebees, and nectar-robbing bumblebees) and hoverflies (Hepper, 1956; Jürgens et al., 1996). The temporal distribution of these floral visitors is clearly disjunct: while moths visit flowers from dusk till dawn (in June in Belgium: 21:30–05:30), with a visitation peak around midnight; bees and flies forage during the daytime, in June from 06:00 the earliest to 21:00 the latest (Vanderplanck, personal observations). The fruit of *S. nutans* is an ovoid capsule that contains small, numerous, grey-blackish and irregularly kidney-shaped seeds (De Bilde, 1973; Jonsell, 2001).

2.2. Pollinator exclusion experiment

Several distinct western and eastern genetic lineages have been identified within *S. nutans* in Europe, which show reproductive isolation (Martin et al., 2016, 2017; Van Rossum et al., 2018). Therefore, the pollinator exclusion experiment was carried out using only one genetic lineage (genetic lineage W1; Martin et al., 2017). An artificial population of *S. nutans* was established using a total of 36 hermaphroditic individuals from the plant collection of the University of Lille. By using an artificial population, we were able to minimize variation among pollination treatments regarding plant size, floral display, relatedness and microhabitat that would otherwise contribute to seed production variation in natural populations (Stone and Olson, 2018). As we did not have enough individuals originating from a unique wild population, we mixed plants from eight wild populations of Belgium and France (genetic lineage W1; Table 1). Pure female individuals as well as female

flowers on hermaphrodite individuals were excluded from the present study since female flowers can lead to pollen limitation (*i.e.* insufficient pollen production) at the population level (Alexander, 1987), an effect that we wanted to avoid in our experimental population. Furthermore, female flowers and female plants are quite rare in natural populations for the genetic lineage W1 (on average 16% of gynomonecious plants – carrying on average a low proportion of female flowers – and 4–15% of female plants in southern Belgium; De Bilde, 1984; Dufay et al., 2010; Van Rossum, unpublished data). All plants were over-wintered for ten weeks during the winter 2017–2018 and then potted in a soil mix (3/4 compost; 1/4 perlite). Plants were placed in a greenhouse at a temperature of 20 °C for ten days and were then acclimatized to outdoor conditions for one month before the start of the experiment. The artificial population was set up in a common garden on the campus of Mons University (location: 50°27′N, 3°57′E, Belgium), outside of the natural range of the species with no wild populations at close proximity.

Pollinator exclusion treatments were conducted using large exclusion cages covered with an insect-proof net (mesh size 920*920 μm) that prevents pollinator access during the day or the night (Fig. A.1A). The 36 hermaphrodite plants were assigned to one of the three following pollinator exposure treatments: (i) diurnal pollination (12 day-pollinated plants, exclusion cages from 21:30–21:45 to 05:45–06:00), (ii) nocturnal pollination (12 night-pollinated plants, exclusion cages from 05:45–06:00 to 21:30–21:45) and (iii) open pollination (12 control plants, never in exclusion cages) (Fig. A.1B). The non-overlapping day-night periods in the experimental population have been established according to the temporal distribution of diurnal and nocturnal visitors (Vanderplanck, personal observations). The plants were distributed among treatments to ensure homogeneity of geographical origins (Table 1) and of flower and bud numbers at the start of the experiment (Table A.1). Artificial lighting was turned off at night during the whole experiment since light pollution can disrupt nocturnal pollination (Macgregor et al., 2017). All plants were watered and moved daily to avoid spatial effects. Five flowers per plant were marked with colored threads at bud stage before the experiment and followed until the end of the receptive phase (*i.e.* wilted petals and dried stigmas). The controlled exposure to pollinators began on June 1st, 2018, during the peak of flowering, and ended on 12 June, when all marked flowers were no longer receptive. Such control for different factors in a single environment will allow for a clearer interpretation of the potential effects of pollinator treatments.

We conducted an insect fauna inventory in the common garden at Mons and carried out observations of flower visitors during the experiment (during one day and one night) to ensure that potential pollinators were active during both day and night periods. Daylight observations were performed focusing on single plants, whereas nocturnal observations were made by actively searching for pollinators across the population under ambient light, since single-plant observations in darkness did not work properly. At the end of the experiment, plants were all moved to a greenhouse for fruit ripening to limit predation (*i.e.* by slugs). Fruits were bagged to prevent seed loss and collected at fruit maturity just after capsule dehiscence, about four weeks after fruit initiation.

Table 1
List of the eight sampled populations of the W1 genetic lineage of *S. nutans*.

Population	Location	Coordinates	Number of plants (open, diurnal, nocturnal pollination)
BZH2	France, Bretagne, Finistère	47°46′N, 3°39′E	3 (1, 1, 1)
BZH5	France, Bretagne, Côtes d'Armor	48°34′N, 2°34′E	3 (1, 1, 1)
BZH7	France, Bretagne, Loire-Atlantique	47°07′N, 1°24′E	3 (1, 1, 1)
BZH9	France, Bretagne, Morbihan	47°35′N, 3°03′E	12 (4, 4, 4)
HOU	Belgium, Wallonia	50°11′N, 5°00′E	2 (0, 1, 1)
MOR	France, Auvergne	45°35′N, 2°52′E	7 (3, 2, 2)
RUN	France, Cévennes	44°23′N, 3°41′E	1 (1, 0, 0)
VIR	Belgium, Wallonia	50°05′N, 4°43′E	5 (1, 2, 2)

2.3. Floral traits and resources

Floral and reward traits constitute cues that pollinators can use to select the plants they will forage on (reviewed in Dötterl and Vereecken, 2010). As such traits are part of the attraction components of pollination syndromes of the *Silene* species (Reynolds et al., 2009), it was mandatory to characterize them and ensure that all pollination treatments were equal to each other in our experimental population. We decided to focus on a subset of traits usually considered in plant-pollinator studies (Reynolds et al., 2009; Leonard et al., 2012; Junker and Parachnowitsch, 2015; Akter et al., 2017; Rusman et al., 2019).

Plant and flowers – During the experiment, the number of flowers was counted every day on each of the 36 plants. At the end of the experiment, the maximum plant height (in cm) was measured from soil level using a measuring tape. For each plant, the following traits were measured to the nearest 0.1 mm with a digital calliper on one randomly chosen marked flower two days after flower opening (stage F2): daytime and nighttime corolla diameter, corolla depth, calyx length and width, as well as length and width of one randomly selected petal (Fig. A.2).

Pollen – At the end of the experiment, one floral bud per plant was collected and immediately dissected. The anthers from the first whorl were used to assess pollen viability according to Peterson et al. (2010). Pollen grains were removed from the anthers and placed on a glass slide with 2–3 drops of Peterson stain solution (10 mL 95% ethanol, 1 mL 1% malachite green in 95% ethanol, 50 mL H_2O, 25 mL glycerol, 5 mL 1% acid fuschin in H_2O, 0.5 mL 1% orange G in H_2O, 4 mL glacial acetic acid, and 4.5 mL H_2O; Peterson et al., 2010), which stains pollen grain cytoplasm in purple and exine in green. A coverslip was used to cover the pollen and Peterson mixture, and sealed using nail polish. Two pollen samples per floral bud were then examined under light microscope at $\times 100$ magnification. Two hundred fifty pollen grains per slide were scored as either viable (purple cytoplasm present) or aborted (empty, only the green exine visible), and pollen viability (%) was calculated as the ratio of viable (*i.e.* purple-stained) pollen grains to the total number of counted pollen grains multiplied by 100. For the estimation of pollen production, the anthers from the second whorl were collected and stored in ethanol at 95% until analysis. Samples were placed at 56 °C overnight to evaporate the solvent before adding 1 mL of distilled water. The anthers were then gently broken by squeezing them with a mounted needle, and sonicated for 10 min to release the pollen. The number of pollen grains was estimated for each sample using a particle counter CASY model TT (Innovatis, Bielefeld). Tubes were vortexed, and 300 μL of the pollen suspension were diluted in 5 mL of pure water CASY ton for cell counter. Each solution was then carefully shaken to ensure homogeneity before analysis. The particle counter sampled three volumes of 400 μL and provided the number of detected particles for the total 1200 μL analyzed for 400 size classes ranging from 0.3 to 120 μm using the software CASY Excel 2.1. A two-fold dilution was used for samples above the upper concentration limit of the particle counter. The total number of pollen grains from the second whorl was obtained from the values provided by the particle counter after correcting for the dilution ratio (Dufay et al., 2010) and then multiplied by 2 for estimating the total number of pollen grains per flower.

Nectar – At the end of the experiment, one floral bud per plant was bagged before flower opening to prevent visits by insects. The nectar was collected on the third day of flower development at 09:00 (stage F3) using a 0.5 μL glass capillary tube (Hirschmann® Laborgerate, Eberstadt, Germany). Nectar volume (in μL) was estimated by measuring the length of the nectar column in the capillary tube. The nectar sugar concentration was measured using a low volume hand-held sugar-refractometer (Eclipse Handheld refractometer, Bellingham & Stanley Ltd, Tunbridge Wells, UK), and is expressed as degrees Brix, which represent the percentage of sucrose per nectar mass (w/w).

2.4. Pollination success and offspring fitness

A total of 77 focal fruits from the initial marked flowers (*i.e.* 180 flowers) were counted and dissected onto round Petri dishes to recover the seeds and the unfertilized ovules that were carefully removed from the central axis of the capsule. Chaff (*i.e.* vegetal and foreign material) was removed using tweezers, and oviposition events from nursery pollinators (mainly *Hadena* and *Perizoma* genera) as well as caterpillar predation were recorded. Petri dishes were then scanned using an Epson Perfection V700 PHOTO Scanner. The total number of unfertilized ovules and of seeds (*i.e.* fertilized ovules, which is an indicator of pollination events) per fruit were directly counted on the pictures. The number of unfertilized ovules and seeds were summed to estimate the total number of ovules in each fruit. Pollination success of individual plants was assessed by means of three variables: fruit set (proportion of marked flowers setting fruit), seed number per fruit and total seed production over the five focal flowers (focal fruits only). Offspring fitness was estimated by measuring seed mass (analytical balance, XT 120A Precisia, d = 0.1 mg) and by testing seed germination. Seed germination was carried out by placing a set of maximum 50 seeds per focal fruit on 1% water agar substrate in plastic Petri dishes of 90 mm diameter. The dishes were placed in a greenhouse at 10/20 °C alternating temperature and a 14/10 h light/dark photoperiod. The criterion for considering that the germination was effective was the visible protrusion of the radicle (≥ 1 mm). Germinated seeds were scored daily and the dishes were regularly randomized. The germination trials were terminated after three weeks, when no additional germination occurred (Fig. A.3). The germination rates per focal fruit and per plant (*i.e.* over all focal fruits of this plant) were calculated as the proportion of seeds that have germinated at the end of the trials. We also used the total number of seeds (quantity component) multiplied by the germination rate (quality component) per plant as a proxy for the plant fitness (Erb, 2018).

2.5. Statistical analyses

To ensure that floral and resource traits (potential cues for pollinators) did not differ among treatments, especially between control and diurnal/nocturnal treatments, general linear models were computed for each measured trait (floral traits, and nectar and pollen variables; Table A.2) with pollination treatment as a fixed effect and plant population as a random factor (GLMM; "glmer" function, R-package "lmerTest"; Kuznetsova et al., 2017). For the ovule number per flower, an individual effect was included as a hierarchical random effect in the model so that the non-independence of the data could be taken into account. A binomial model was used for pollen viability, with an observation-level random effect added to the model to account for overdispersion (*i.e.* each data point received a unique level of random effect that modelled the extra-parametric variation present in the data; Harrison, 2014). A Gaussian distribution model was used for the other traits (see Table A.2). Plant height and nectar amount were log-transformed to achieve normality of the residuals. Daytime corolla diameter, corolla depth and pollen-ovule ratio were rank-transformed to normality of residuals ("rntransform" function, R-package "GenABEL"). *P* values were corrected for multiple testing using Tukey contrasts ("glht" function, R-package "multcomp"; Hothorn et al., 2008). Difference in corolla diameter between daytime and nighttime was analyzed using a paired *t*-test in order to determine whether *S. nutans* hermaphrodite flowers are more conspicuous in the nighttime than in the daytime.

To test for differences in pollination success (fruit set, seed number per fruit, total seed production over the five focal flowers), offspring fitness (seed mass, seed germination) and plant fitness proxy (seed number per plant multiplied by the germination rate per plant) among pollination treatments, we performed GLMMs with pollination treatment as a fixed effect and plant population as a random effect. Fruit set, total seed number, mean seed mass, germination rate and plant fitness

data were analyzed at plant level, whereas seed number per fruit was analyzed at fruit level (*i.e.* data points were fruits), with individual plants included as a hierarchical random effect in the model so that the non-independence of the data can be taken into account. Total seed number per plant, seed number per fruit, mean seed mass and plant fitness were analyzed using models with a Gaussian error structure (*i.e.* normally distributed residuals). Fruit set was analyzed using a binomial model with the number of fruits (successes) and the number of un-fertilized flowers (failures) as a bivariate response. A binomial model was also used for germination rate (*i.e.* number of seedlings and number of ungerminated seeds as a bivariate response) and an observation-level random effect was added to the model to account for overdispersion (Harrison, 2014). When a significant effect was found, multiple pair-wise comparison tests were performed using Tukey contrasts to de-termine how pollination treatments significantly differed from each other.

To study the linear relationships between (i) seed number per fruit and mean seed mass, (ii) seed number per fruit and germination rate per fruit, and (iii) mean seed mass and germination rate per fruit (fruit-level data); we computed three GLMMs with a Gaussian error structure and plant population as a random effect. Models incorporated in-dividual plants as a hierarchical random effect. Conditional ($R^2_{(c)}$, the variance explained by both fixed and random factors) and marginal ($R^2_{(m)}$, the variance explained by fixed factors only) coefficients of de-termination were calculated using the "r.squaredGLMM" function ("MuMIn" R-package). Data of germination rate per fruit were arcsin transformed and all residuals were normally distributed (Shapiro-Wilk test, $p > 0.05$ for all models). All analyses were performed in R version 3.4.0 (R Core Team, 2017).

3. Results

We observed several active diurnal (mainly bees and hoverflies) and nocturnal (mainly moths) floral visitors in the artificial population during the experiment (Table 2). Flowers of *S. nutans* were legitimately visited by both diurnal and nocturnal visitors and were only robbed by *Bombus terrestris*, which also undertook legitimate visits (the visit was considered as legitimate when a visitor touched the stigmas, and ille-gitimate when it did not). The observed bumblebees predominantly visited near-neighbor plants and tended to visit the flowers sequentially on the inflorescence, from the bottom to the top. On the contrary, moths frequently bypassed neighbors and appeared to fly greater dis-tances between plants. No species appeared in both diurnal and noc-turnal flower visitor assemblages (Table 2).

All measured traits (*i.e.* floral traits and resources) were similar among pollination treatments ($p > 0.05$), except the daytime corolla diameter that was significantly larger for the flowers of the diurnal pollination treatment than those of the nocturnal one (Table A.2). Be-cause flowers of the night-pollinated plants were encaged during day, this difference could not be perceived by pollinators. Regardless of the pollination treatment, the nighttime corolla diameter of hermaphrodite flowers was about three times larger than the daytime one (paired *t*-test: $t = -21.65$, $df = 33$, $p < 0.001$; Fig. 2).

All pollination treatments resulted in fruit and seed production (Table A.3 and Fig. 3). However, the fruit set, the seed number per fruit and the total seed production over the 5 focal flowers were all affected by pollination treatment ($\chi^2 = 33.65$, $df = 2$, $p < 0.001$; $\chi^2 = 14.98$, $df = 2$, $p < 0.001$; $\chi^2 = 13.44$, $df = 2$, $p = 0.001$; respectively) with significantly lower values in day-pollinated plants compared to the plants of the two other treatments (pairwise comparisons, $p < 0.05$). Note that differences in seed number per fruit were due to differences in rates of fertilization as ovule number per flowers was similar among treatments (Table A.2). For all of variables, no significant difference was found between open and nocturnal pollination treatments (Table A.3 and Fig. 3; pairwise comparison tests, $p > 0.05$). The magnitude of the treatment effect was extremely strong, with on average a number of

Table 2
List of diurnal and nocturnal insects observed in the common garden at Mons, Belgium in 2018. Species in bold were recorded as floral visitors of *Silene nutans*.

Diurnal insects	Nocturnal insects
Coleoptera	Lepidoptera
Cantharidae	*Anania hortulata* (Crambidae)
Cetonidae	*Autographa gamma* (Noctuidae)
Chrysomelidae	*Chrysoteuchia* cf. *culmella* (Crambidae)
Cucurlionidae	*Cucullia umbratica* (Noctuidae)
Oedemera sp. 1 (Oedemeridae)	*Eupithecia linariata* (Geometridae)
Oedemera sp. 2 (Oedemeridae)	*Eupithecia* cf. *tripunctaria* (Geometridae)
	Idaea fusconevosa (Geometridae)
Diptera	*Anania hortulata* (Crambidae)
Conopidae	*Autographa gamma* (Noctuidae)
Episyrphus balateus (Syrphidae)	*Chrysoteuchia* cf. *culmella* (Crambidae)
Eupeodes luniger (Syrphidae)	*Cucullia umbratica* (Noctuidae)
	Eupithecia linariata (Geometridae)
Hymenoptera	
Andrena gr. *flavipes* sp. 1 (Andrenidae)	*Eupithecia* cf. *tripunctaria* (Geometridae)
Andrena sp. 2 (Andrenidae)	*Scopula* sp. (Geometridae)
Andrena sp. 3 (Andrenidae)	*Yponomeuta* sp. (Yponomeutidae)
Anthidium manicatum (Megachilidae)	
Apis mellifera (Apidae)	
Athalia sp. (Tenthredinidae)	
Bombus horthorum (Apidae)	
Bombus lapidarius (Apidae)	
Bombus pascuorum (Apidae)	
Bombus terrestris (Apidae)	
Chelostoma sp. (Megachilidae)	
Chrysis sp. (Chrysididae)	
Colletes sp. (Colletidae)	
Eumenidae	
Ichneumon sp. 1 (Ichneumonidae)	
Ichneumon sp. 2 (Ichneumonidae)	
Lasioglossum sp. (Halictidae)	
Macropis europaea (Mellitidae)	
Megachile sp. 1 (Megachilidae)	
Megachile sp. 2 (Megachilidae)	
Nomada sp. (Apidae)	
Thyreus orbatus (Apidae)	
Vespula sp. (Vespidae)	
Lepidoptera	
Aglais urticae (Nymphalidae)	
Araschnia levana (Nymphalidae)	
Pararge aegeria (Nymphalidae)	
Pieris sp. (Pieridae)	
Polyommatus icarus (Lycaenidae)	

seeds per fruit about six times lower for diurnal pollination treatment than for the nocturnal ones. No oviposition events were recorded among the focal fruits or even outside (*i.e.* non-focal fruits) at the end of the experiment, regardless of the pollinator exclusion treatment. In the same way, no predation events were recorded, except for some non-focal flowers (*i.e.* predation by slugs).

Given the strong negative correlation ($r = -0.58$, $p < 0.001$) be-tween the seed number per fruit and the mean seed mass (Fig. 4A), seeds produced by plants from the diurnal treatment were significantly heavier than seeds from plants of the nocturnal ($p < 0.001$) and open ($p < 0.001$) treatments (Table A.3 and Fig. 3D). Although mean seed mass and germination rate per fruit were positively correlated ($r = 0.58$, $p < 0.001$; Fig. 4C), no significant relationship was found between seed number and germination rate per fruit ($p = 0.993$; Fig. 4B). Although seeds produced during the day tended to germinate better – possibly because of the extremely low number of fruits and seeds produced in this treatment – the effect of pollination treatment on germination rate was not significant ($\chi^2 = 5.88$, $df = 2$, $p = 0.053$; Table A.3 and Fig. 3E).

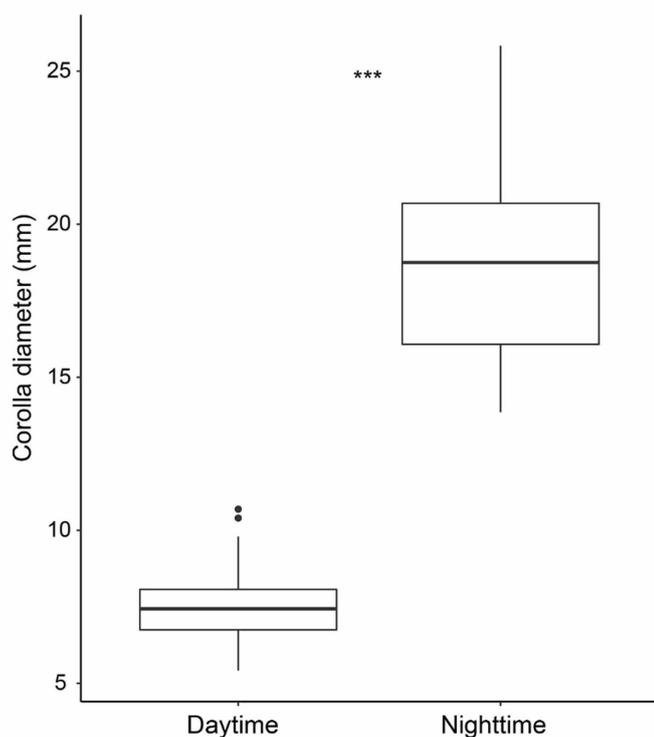Overall, plants from the nocturnal and open pollination treatments

Fig. 2. Box plots of the corolla diameter of flowers during the daytime and the nighttime in the *Silene nutans* experimental population.

displayed significantly higher values of fitness proxy than plants from the diurnal treatment (χ^2 = 11.99, df = 2, p = 0.002; Fig. 3F), mainly because of the higher seed production.

4. Discussion

Pollination occurred during both day- and nighttime, since at least a proportion of flowers from diurnal and nocturnal pollination treatments set fruits and most of the known diurnal pollinators of *S. nutans* (Hepper, 1956; Jürgens et al., 1996) occurred in our experimental population. However, fruit and seed production was much higher in plants pollinated by nocturnal visitors compared to plants pollinated during the day. These results differ from previous studies on other *Silene* species, which have found similar (*S. latifolia*; Scopece et al., 2018; *S. vulgaris*; Stone and Olson, 2018) or higher (*S. ciliata*; Giménez-Benavides et al., 2007) fruit production during the day, but with a higher seed number per fruit produced at night. Such production patterns likely resulted from diurnal visitors displaying high flower visitation rates, but a lower efficiency in effective pollination compared to the nocturnal ones (Giménez-Benavides et al., 2007; Scopece et al., 2018). The difference in fruit production in our experimental system thus suggests that efficient flower visitations (*i.e.* with sufficient conspecific pollen deposited to initiate fruit development) by diurnal visitors are quite rare compared to nocturnal ones. This could be explained by the typical moth pollination syndrome of *S. nutans* (Jürgens et al., 1996, 2002; Witt et al., 1999), with flowers being more inconspicuous in the daytime (reduced corolla diameter due to rolled petals; Jürgens et al., 1996; present study) with less intense scent emission and reduced nectar production (Witt et al., 1999; Jürgens et al., 2002).

Regarding seed production per fruit, consistently with previous studies led on other *Silene* species, our results suggest that diurnal visitors may be less efficient pollinators than the nocturnal ones on *Silene nutans*, regarding amounts of pollen removed and deposited (*i.e.* "ugly" pollinators; Thomson and Thomson, 1992). For instance, bees are able to specifically pass over the stigmas during flower visitation and they remove large amounts of pollen, so that pollen transfers are

minimized (Müller, 1996; Larsson, 2005; see also "pollen robbers" Inouye, 1980). They can also obtain nectar through perforations in the corolla tube of flowers ("nectar robbers"; Inouye, 1980; Maloof and Inouye, 2000). Such illegitimate visits have been observed in our experimental population and may reduce the reproductive success of a plant directly, by damaging flowers, or indirectly, by producing changes in nectar quality and quantity that affect legitimate pollinator behavior (Gonzalez-Gomez and Valdivia, 2005). Moreover, bee females carefully groom their body after each flower visit and transfer the pollen to specialized, external carrying structures (scopae, including corbiculae), making it unavailable for flower pollination (Thorp, 1979; Westerkamp, 1996). Among the diurnal insects recorded as floral visitors of *S. nutans* in our experimental system, flies can also carry substantial amounts of pollen (Yeboah Gyan and Woodell, 1987; Kearns, 1992) and may efficiently contribute to the pollination of some plant species (e.g. the syrphid *Eristalis tenax* on *Cistus libanotis*, Talavera et al., 2001; muscid flies on *Rhododendron ferrugineum*, Escaravage and Wagner, 2004). According to the observations, Lepidoptera (no observation) and Coleoptera (only one observation) were only occasional visitors of *S. nutans* and have certainly a negligible impact on the plant fitness. Contrary to bees and flies, moths remove less pollen from a flower and groom less of it off their bodies. They can then deliver significant amounts of pollen to stigmas, which can result in a higher pollination success (Pettersson, 1991b; Willmott and Burquez, 1996).

Combining fruit set and seed number per fruit over the five focal flowers resulted in an extremely low total seed production in plants exposed during the day only, without any compensation between fruit set and seed number. In our system, the effectiveness of pollinator guilds on *S. nutans* is consistent with the described moth pollination syndrome (Jürgens et al., 1996, 2002; Witt et al., 1999). Several other studies have found a difference in pollination effectiveness between nocturnal and diurnal visitors (e.g. Ortiz et al., 2000; Luo et al., 2011; Ortega-Baes et al., 2011; Amorim et al., 2012), including in other species of the *Silene* genus (Young, 2002; Barthelmess et al., 2006). However, numerous plant species rather display a mixed pollination strategy, with the insect abundances sometimes offsetting their performance as pollinators (Cruden, 1973; Bertin and Willson, 1980; Haber and Frankie, 1982; Jennersten, 1988; Jennersten and Morse, 1991; Guitian et al., 1993; Miyake and Yahara, 1998; Prieto-Benitez et al., 2016). In several of these cases, among populations and/or years, variation in insect densities, plant densities or abiotic parameters lead to some variation in the relative contribution of nocturnal vs. diurnal pollinators within the species range (e.g. Holland and Fleming, 2002; Barthelmess et al., 2006; Reynolds et al., 2009; Scopece et al., 2018). In such case, mixed pollination may lead to some bet-hedging strategy, allowing significant levels of pollination in several environmental conditions. Since our study was conducted in a common garden experiment, in one location only, our results may not accurately reflect what occurs in natural populations of *Silene nutans*. In particular, we did not record the occurrence of *Silene* nocturnal nursery pollinators (especially *Hadena* and *Perizoma* moths), of which caterpillar fruit and seed predation may offset their pollination efficiency (*i.e.* high pollination success but reduced number of seeds available for dispersal; Van Rossum, 1996; Scopece et al., 2018).

Our data suggest a trade-off between seed mass and seed number, which has also been reported in natural populations of *S. nutans* W1 genetic lineage in Belgium (Van Rossum, 1996). The resulting higher seed mass produced by the diurnal pollination treatment did not seem to impact the germination rate in our experiment (however based on a low number of seeds). No effect of seed mass on germination rate has also been found for intra-lineage crosses involving the studied populations (Van Rossum et al., unpublished data). This contrasts with results found in *S. ciliata* in which any shortage of the specialized nocturnal pollinator may not put the plant in danger, since seeds produced by co-pollinators are of higher quality (Giménez-Benavides et al., 2007). Other factors may also influence the seed quality such as the
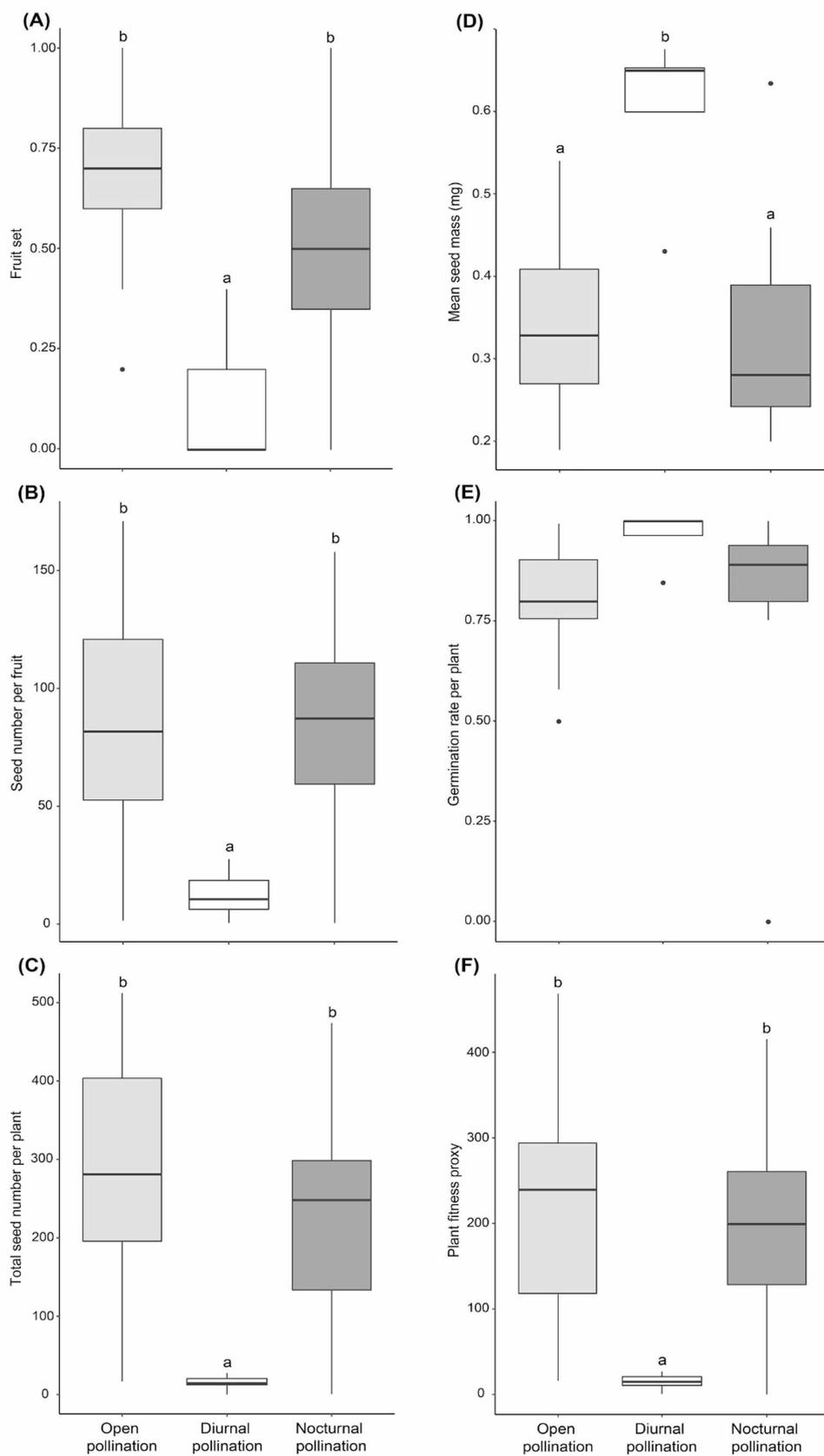
Fig. 3. Box plots of the components of pollination success, offspring and plant fitness in the pollination exclusion experiment: (A) fruit set, (B) seed number per fruit, (C) total seed number per plant, (D) seed mass, (E) seed germination and (F) plant fitness using the total number of seeds (quantity component) multiplied by the germination rate (quality component) per plant as a proxy. Different letters indicate significant differences (p < 0.05) between two pollination treatments.
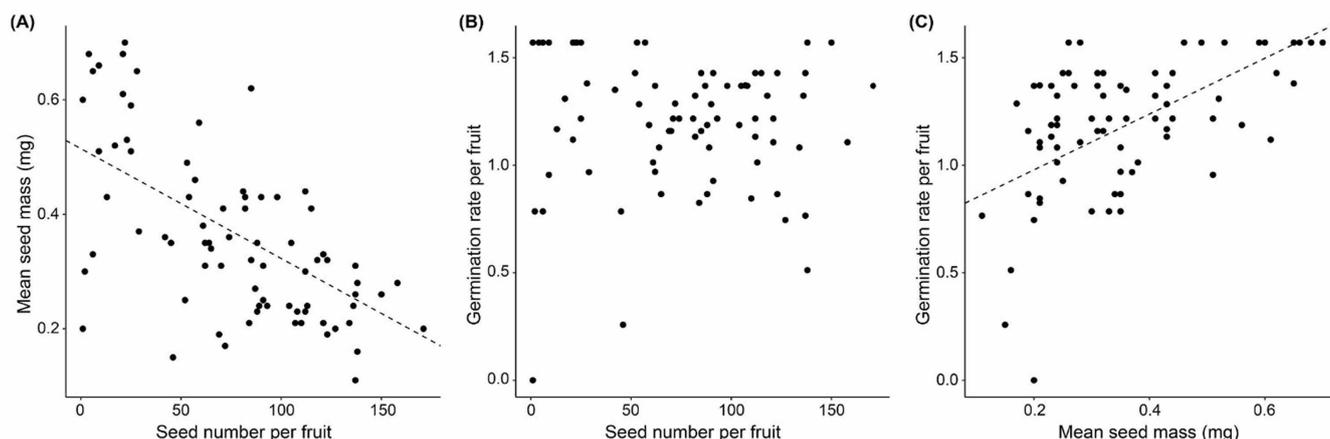
Fig. 4. Relationships between (A) the seed number per fruit and mean seed mass (slope = −0.002, p < 0.001, $R^2_{(m)}$ = 0.33, $R^2_{(c)}$ = 0.59), (B) the seed number per fruit and seed germination per fruit (arcsin transformed) (p = 0.993), and (C) mean seed mass and seed germination per fruit (arcsin transformed) (slope = 1.298, p < 0.001, $R^2_{(m)}$ = 0.33, $R^2_{(c)}$ = 0.62) in the *Silene nutans* experimental population (fruit level data).

proportion of outcrossed capsules. Although the low seed production in the diurnal treatment prevented us to investigate the selfing rate in our experimental population, we may expect that typical foraging behaviors and flight distances of diurnal visitors might result in higher levels of inbreeding in *S. nutans* (Schmitt, 1980; Herrera, 1987; Barthelmess et al., 2006). Significant levels of inbreeding depression have been found in *S. nutans* at both early (reduced seed quality and germination) and late life stages (floral traits and reproductive success, plant size and survival rate: Dufay et al., 2010; Thiele et al., 2010). Restricted pollen flow and increased selfing in diurnal pollination may thus result in a further decrease of offspring quality. Although our study did not show evidence for a reduced quality (early stages) of seeds produced during the day, investigating the variation of selfing rate between the two modes of pollination and its potential impact on offspring quality constitutes a stimulating perspective of the current study.

Regarding the plant fitness proxy in our system (combination of seed number and seed quality), no difference was highlighted between open and nocturnal pollination treatments, and no obvious compensation was found between seed number and seed quality in the diurnal pollination treatment. The relative contribution of diurnal pollinators to reproductive success of *S. nutans* is clearly weak and even negligible in our experimental system, which confirms its described moth pollination syndrome (Jürgens et al., 1996, 2002; Witt et al., 1999). Such specialized pollination strategy of *S. nutans* may result in population vulnerability in some conditions; for instance, with similar diurnal and nocturnal pollinator guilds to our experimental system, artificial lighting at night might reduce visits of nocturnal pollinators (Macgregor et al., 2017). However, caution has to be paid since this experimental outcome (i.e. nocturnal specialized pollination strategy) could differ according to the pollinator guilds visiting the populations of *S. nutans*: a change in the composition of the diurnal visitors might increase their importance to the system while the occurrence of nocturnal nursery pollinators despite increasing pollination success might reduce the contribution of nocturnal pollinators to plant reproductive success. Additional studies of the relative contributions of various pollinators to reproductive success in *S. nutans* are needed across its range and multiple years to make the broader generalization that this pollination system shows a nocturnal specialization.

Author contributions

MD, PT and IDC conceived the idea. MV conceived the experimental design that supports the manuscript with the advice of FVR, MD, PT, IDC and EL. MV conducted the experimentation and laboratory work (i.e. pollen and nectar analyses, pollen and seed counts, germination trials). MV conducted the statistical analysis and wrote the draft; and MD, PT, FVR and IDC critically reviewed and contributed to the final manuscript. All authors have approved the final article.

Declaration of competing interest

The authors declare no conflict of interest.
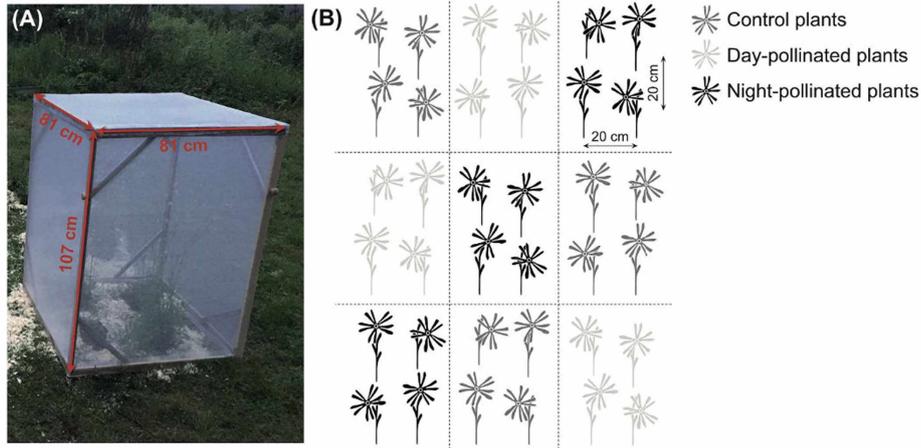
Acknowledgments

Appendices

Fig. A.1. Exclusion cage (A), and schematic representation of the experimental design (B). Three exclusion cages were constructed using a wooden structure (81*81*107 cm) covered by an insect-proof net (mesh size 920*920 μm). The *S. nutans* experimental population consisted of 36 hermaphrodite plants. Twelve plants served as control in open pollination (grey symbols), twelve plants were assigned to nocturnal pollination (black symbols) and twelve plants to diurnal pollination (creamy-white symbols) treatment. Plants were arranged using a randomized complete block design with nine blocks containing four plants each (*i.e.* three blocks per pollination regime). Within each block, plants were spaced 20 cm apart. The relative positioning of the treatments was changed every day, and the relative positioning of plants within treatment was randomly assigned and moved daily to control for handling and spatial effects.

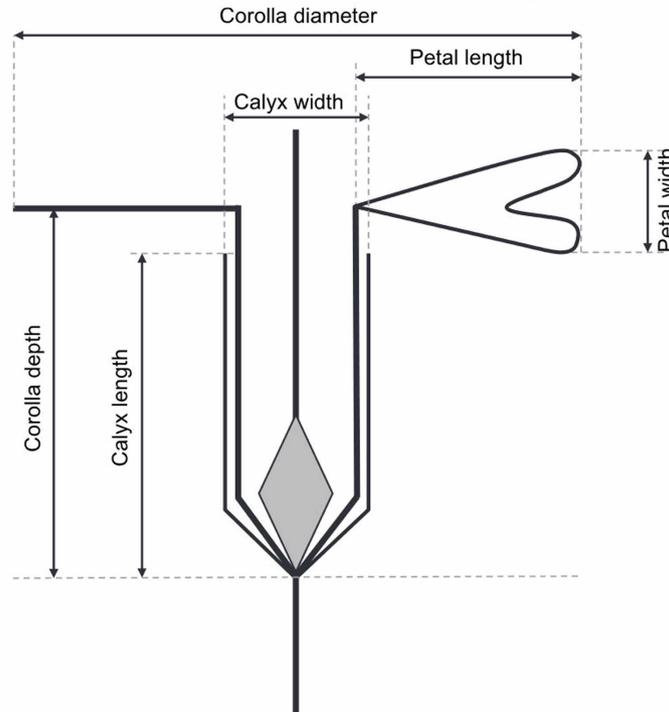**Fig. A.2.** Schematic cross-section of a hermaphrodite *Silene nutans* flower illustrating measurement of floral traits. Only one of the styles and petals are illustrated here (adapted from Giles et al., 2006).

Giles, B.E., Pettersson, T.M., Carlsson-Graner, U., Ingvarsson, P.K., 2006. Natural selection on floral traits of female Silene dioica by a sexually transmitted disease. New Phytol. 169, 729–739.
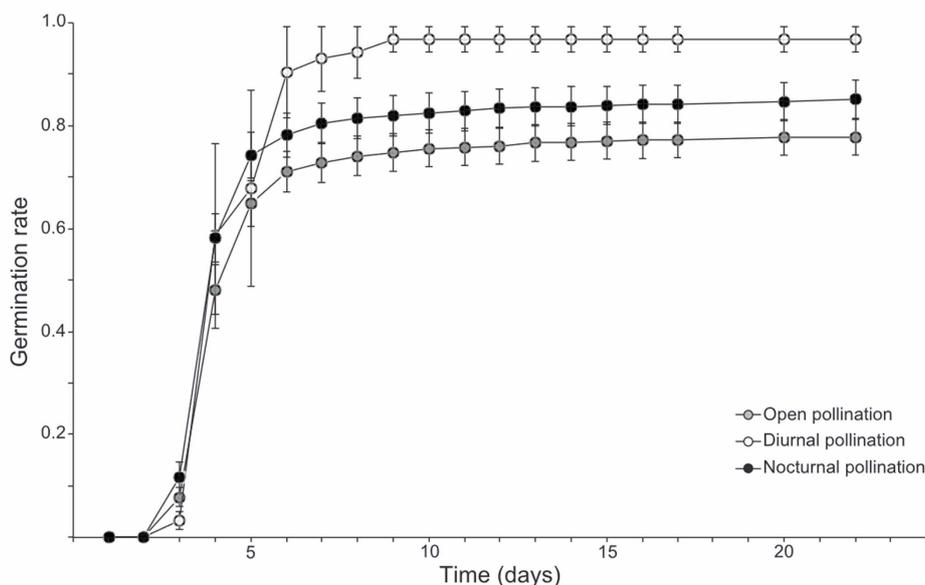
Fig. A.3. Seed germination rate (mean ± SE) over time for seeds the three pollination treatments (open, diurnal and nocturnal pollination) in the *Silene nutans* experimental population at Mons, Belgium in 2018.

Table A.1
Comparison of the total number of floral buds and flowers (means ± SD) among pollination treatments at the beginning of the experiment (n = 12 plants per treatment). Poisson regression model for count data was used.

Pollinator exclusion treatment	Number of floral buds	Number of flowers
Open pollination	27 ± 24	11 ± 14
Diurnal pollination	27 ± 21	9 ± 11
Nocturnal pollination	27 ± 25	9 ± 9
Statistics	χ^2 = 0.002, df = 2, p = 0.999	χ^2 = 2.519, df = 2, p = 0.284

Table A.2
Plant, floral and resource traits (means ± SD) for the three treatments (diurnal, nocturnal and open pollination) in the *Silene nutans* experimental population. Different letters indicate significant differences (p < 0.05) between two pollination treatments. Significant differences are in bold; n, sample size (number of individuals, except for the ovule number per flower, which was measured for each flower).

Floral traits	Open pollination (n)	Diurnal pollination (n)	Nocturnal pollination (n)	Statistics
Plant height (cm)	42.50 ± 7.22 (12)	40.60 ± 14.13 (12)	44.67 ± 9.74 (12)	X^2 = 2.11, df = 2, p = 0.349
Maximum number of flowers	30.17 ± 24.14 (12)	33.25 ± 22.76 (12)	29.75 ± 25.70 (12)	χ^2 = 0.34, df = 2, p = 0.844
Sex ratio at flowering peak	1.41 ± 1.20 (12)	0.72 ± 0.41 (12)	1.04 ± 0.67 (12)	χ^2 = 3.79, df = 2, p = 0.150
Corolla diameter (day) (mm)	7.58 ± 1.14 [ab] (11)	8.06 ± 0.93 [b] (12)	7.22 ± 1.45[a] (11)	X^2 = 7.23, df = 2, p = 0.027
Corolla diameter (night) (mm)	19.27 ± 2.51 (11)	18.39 ± 3.67 (12)	18.29 ± 3.18 (11)	X^2 = 0.65, df = 2, p = 0.724
Corolla depth (mm)	14.50 ± 1.00 (11)	14.92 ± 0.94 (12)	15.23 ± 2.09 (11)	X^2 = 1.80 df = 2, p = 0.406
Calyx width (mm)	2.99 ± 0.38 (11)	2.82 ± 0.25 (12)	2.63 ± 0.68 (11)	χ^2 = 1.98, df = 2, p = 0.372
Calyx length (mm)	11.24 ± 0.85 (11)	11.11 ± 0.76 (12)	10.77 ± 0.87 (11)	χ^2 = 1.88, df = 2, p = 0.390
Petal width (mm)	6.25 ± 0.89 (11)	5.67 ± 1.40 (12)	5.80 ± 1.46 (11)	χ^2 = 1.26 df = 2, p = 0.533
Petal length (mm)	11.21 ± 1.08 (11)	10.59 ± 1.44 (12)	10.30 ± 1.11 (11)	χ^2 = 2.92, df = 2, p = 0.232
Pollen grain number	17,267 ± 6759 (11)	16,657 ± 5143 (11)	18,706 ± 5636 (11)	χ^2 = 0.70, df = 2, p = 0.706
Pollen viability (%)	82.80 ± 4.00 (11)	81.05 ± 9.65 (11)	80.73 ± 7.78 (11)	χ^2 = 0.07, df = 2, p = 0.964
Ovule number per flower	148 ± 50 (41)	156 ± 34 (6)	144 ± 20 (30)	χ^2 = 0.22, df = 2, p = 0.896
Pollen-ovule ratio	149 ± 156 (11)	121 ± 44 (5)	123 ± 44 (10)	χ^2 = 0.41, df = 2, p = 0.816
Nectar amount (μL)	0.58 ± 0.51 (11)	0.66 ± 0.43 (11)	0.65 ± 0.46 (11)	χ^2 = 0.19, df = 2, p = 0.910
Nectar concentration (%)	32.10 ± 6.16 (11)	27.74 ± 7.23 (11)	30.05 ± 8.60 (11)	χ^2 = 2.05, df = 2, p = 0.359

Table A.3
Pollination success and offspring fitness variables for the three pollination treatments (open, diurnal and nocturnal pollination) in the *Silene nutans* experimental population.

Variables	Open pollination			Diurnal pollination			Nocturnal pollination		
	Mean ± SD	Min – max	n	Mean ± SD	Min – max	n	Mean ± SD	Min – max	N
Focal fruits per plant	3 ± 1	1–5	12	1 ± 1	0–2	12	3 ± 1	0–5	12

Table A.3 (*continued*)

Variables	Open pollination			Diurnal pollination			Nocturnal pollination		
	Mean ± SD	Min – max	n	Mean ± SD	Min – max	n	Mean ± SD	Min – max	N
Fruit set	0.68 ± 0.23	0.2–1	12	0.10 ± 0.13	0–0.4	12	0.50 ± 0.29	0–1	12
Seed number per plant	280 ± 160	17–512	12	16 ± 10	1–28	5	226 ± 137	1–474	11
Seed germination per plant	0.80 ± 0.14	0.5–0.99	12	0.96 ± 0.07	0.85–1	5	0.81 ± 0.28	0–1	11
Seed number per fruit	82 ± 46	2–171	41	13 ± 10	1–28	6	83 ± 36	1–158	30
Seed mass per capsule (mg)	24.4 ± 12.2	0.6–49.7	41	8.1 ± 6.7	0.6–18.2	6	25.4 ± 11.8	0.2–52.2	30
Mean seed mass (mg)	0.3 ± 0.1	0.1–0.7	41	0.6 ± 0.1	0.4–0.7	6	0.3 ± 0.1	0.2–0.7	30

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