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ORIGINAL ARTICLE

Pollination biology and breeding system in five nocturnal species of *Oenothera* (Onagraceae): reproductive assurance and opportunities for outcrossing

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Abstract
The capacity to produce seed, both by selfing and outcrossing, or mixed mating strategies, is considered a mechanism for overcoming unpredictable pollinator availability. In the present study, we investigate breeding system, insect visitations and the role of insect visitors in the pollination of five species of *Oenothera* subsect. *Oenothera*. Field experiments showed that autonomous selfing occurs at bud stage, prior to the opening of the flower. Control flowers showed similar seed set to hand-pollinated flowers, whereas emasculated flowers and those subject to open pollination set fewer seed. These results are consistent with the hypothesis that the investigated *Oenothera* exhibit a great capacity for autonomous selfing and that selfing is selected in order to provide reproductive assurance. Although flowers were visited mostly by nocturnal lepidopterans, these insects did not precipitate pollination and are thus considered nectar thieves. Conversely, analysis of pollen loads and behavior during foraging by diurnal insect visitors revealed that honeybees and bumblebees are the probable pollinators. We conclude that production of flowers capable of autonomous selfing at bud stage, followed by anthesis and opportunities for outcrossing, probably improves the invasive potential of these *Oenothera* in Europe, together with a rapid increase in their populations, even when pollinators are scarce.

Keywords Bumblebees · Plant mating systems · Pollination · Prior selfing · Self-fertilization · Stigma receptivity

Introduction

Most angiosperms depend on animals for pollination, and zoogamy appears to be an essential aspect of successful flowering plant reproduction (Lloyd 1980; Charlesworth and Charlesworth 1987). Evolutionary shifts in flowering plants have led to various mating patterns (Cruden 1977), and the most prevalent trend in the evolution of angiosperm reproduction is a transition from outcross to self-pollination (Lloyd 1992; Charlesworth et al. 1990). Theoretically, such plants should evolve toward either complete self-fertilization or outcrossing (Lande and Schemske 1985; Charlesworth et al. 1990). Studies of selfing rates in natural populations, however, have indicated that mixed mating systems, which involve both selfing and outcrossing, may be more common than is predicted by models (Vogler and Kalisz 2001; Fornoni et al. 2004; Goodwillie et al. 2005). It is generally accepted that one of the most significant factors responsible for the maintenance of a mixed mating system within a population is natural selection for reproductive assurance (Darwin 1876; Baker 1955). The latter model involves strategies which confer fitness advantages that outweigh the disadvantages generally associated with inbreeding and reduced genetic diversity of progeny (Lande and Schemske 1985). Consequently, increased self-fertilization is favored in situations where outcrossing sexual reproduction is limited, for example owing to the scarcity of pollinators and/or mates, or intense competition between individual plants for the service of pollinators (Wolfe and Barrett 1988; Eckert 2002; Kalisz and Vogler 2003; Fenster et al. 2004; Kalisz et al. 2004; Munoz et al. 2015).

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Mixed mating within a population may occur in a variety of ways and/or at different levels. For example, populations may comprise either self-compatible and/or self-incompatible individuals, resulting in a genetically based selfing rate polymorphism (Stone 2002). Besides this, species may also exhibit heteromorphic flower systems, for example cleistogamous (completely selfing) and chasmogamous flowers (involving both outcrossing and selfing, Masuda et al. 2004). The most common system, however, is where individuals possess a single type of flower, and therefore, their fruit contains selfed, outcrossed or a mixture of both progeny types (Schoen and Brown 1991). The selfing that occurs as a consequence of mixed mating may take place within individual flowers (autogamy) or between flowers (geitonogamy) on a particular plant, and within-flower selfing can be either autonomous or vector mediated (Goodwillie et al. 2005 and references therein). Furthermore, mixed mating may occur as a result of one of three modes of autonomous selfing, based on the stage at which outcross pollen is received, namely: prior (before anthesis), competing (during anthesis) and delayed (at late anthesis; Schoen and Brown 1991; Lloyd 1992).

The genus *Oenothera* L. (evening primroses), one of the most studied and complex groups of species in the Onagraceae, has long served as a model system that integrates ecology, evolution and genetics of species interactions (Raven 1988; Johnson 2011). Indeed, species of *Oenothera* have been widely used as experimental plants in studies involving self-incompatibility, chloroplast function, complex heterozygosity and interactions between the genome and plastome. Consequently, *Oenothera* has played an important role in the modern synthesis of evolutionary biology (Johnson 2011; Golczyk et al. 2014). Recent molecular phylogeny work has clarified relationships within *Oenothera*, in particular that of genera that were once segregated: *Gaura*, *Calylophus* and *Stenosiphon* (Wagner et al. 2007 and references therein). Studies of *Oenothera* have revealed considerable variation in pollination and breeding systems; i.e., some taxa are self-incompatible and outcrossed (e.g., *O. greggii* and *O. albicaulis*; Gregory 1964; Theiss et al. 2010), whereas others are self-compatible and autogamous (e.g., *O. sessilis* and *O. simulans;* Krakos and Fabricant 2014; Krakos et al. 2014). According to Raven (1979), hawkmoth pollination is predominant in *Oenothera*, whereas pollination by bees has been documented for sections *Hartmannia* and *Kneiffia*.

In the present study, our analyses focus on five species of *Oenothera* assigned to section *Oenothera* and subsection *Oenothera* (subsect. *Oenothera* hereafter), which differ from other taxa in terms of origin, European distribution and status in Poland (alien, native; sensu Rostański 1985 and Rostański et al. 2010). Previous studies of this subsection indicate that almost all species are self-compatible; however, both self-compatible and self-incompatible individuals have been documented for a population of *O. grandiflora* in Alabama (Stubbe and Raven 1979). The most prominent and typical characteristic of subsect. *Oenothera* is the evolution of autogamy (Dietrich et al. 1997). Thus, based on the classification of breeding systems proposed by Cruden (1977), these species can be considered 'obligately autogamous.' According to Raven (1979), a reduction in flower parts and in the quantities of floral rewards offered has occurred in highly autogamous *Oenothera* as a consequence of shifts from energy-rich pollination systems to less energy-rich systems. More recently, however, it has been demonstrated that several species of subsect. *Oenothera* occurring in Poland produce copious amounts of floral nectar (up to 26 mg/flower), and nectar production has been related to the preferences of floral insect visitors observed in the field (Antoń et al. 2017). Although many papers have been published on members of subsect. *Oenothera*, detailed studies of pollination and reproductive biology have, in fact, been conducted only on *O. elata* in South America (Gregory 1964) and *O. glazioviana* in Japan (Kawano et al. 1995). These showed that both species are mostly hawkmoth pollinated. Even so, a substantial amount of autogamy has also been observed. By contrast, a preliminary survey of floral visitors in members of subsect. *Oenothera* in southeastern Poland (Antoń 2015; Antoń et al. 2017) revealed that hawkmoths do not visit these species. Instead, both nocturnal and diurnal insects (including moths and bees) frequently visit flowers of *Oenothera*, possibly indicating a more generalist pollination syndrome.

To the best of our knowledge, no detailed studies of the pollination biology and breeding systems of species assigned to subsect. *Oenothera* growing in Europe have been undertaken to date. As a result, these species present a great paradox—namely, that despite being as well known both genetically and biologically as almost any other group of plants, the study of much of their pollination and reproductive biology remains neglected. Given that much of the information available for species of subsect. *Oenothera* is contradictory, and given their significance as both crop plants (e.g., in the pharmacological industry; Bosisio 1990) and European invasive species (Mihulka et al. 2003; Tokhtar and Groshenko 2014; Rostański and Verloove 2015), an investigation of their pollination biology and breeding systems is long overdue. In order to address this, we employed four approaches, namely: (i) pollinator exclusion and hand-pollination experiments, (ii) emasculation experiments, (iii) spectrum and behavior analysis of floral visitors and (iv) identification of putative pollinator.

Materials and methods

Study site and plant species

The survey took place at the Botanical Garden of the Maria Curie-Skłodowska University in Lublin, SE Poland (51°15′44′N, 22°30′48′E), during the period 2013–2015. Five *Oenothera* species differing in their geographical distribution and status in Poland (sensu Rostański 1985; Rostański et al. 2010) were investigated. These were: *O. casimiri* Rostański, *O. flaemingina* Hudziok, *O. nuda* Renner ex Rostański, *O. paradoxa* Hudziok and *O. rubricaulis* Kleb. These *Oenothera* species are biennials, and therefore, each year, prior to investigations, experimental plants were established as follows. Seeds produced in 2012 from natural populations established in SE Poland (i.e., in Lublin and Mielec) were sown each year onto a light soil substrate at the end of April for the period 2012–2014. Subsequently, seedlings selected randomly were planted out each September in experimental plots (approx. 2 m^2 in two replicates for each species). During the investigations, the plants were grown each year on loess soil at pH 6–7, at a site fully exposed to the sun.

The genus *Oenothera* currently comprises about 145 species assigned to 18 sections native to North, Central and South America. Of these, about 60 taxa have been reported to occur in the flora of Poland (Rostański and Tokarska-Guzik 1998; Rostański et al. 2004; Wagner et al. 2007; Rostański et al. 2010). Flowers of the investigated species produce both pollen and nectar as floral rewards. Floral nectar is exuded by secretory structures (nectaries) located at the base of a long, tubular hypanthium (Antoń et al. 2017). Pollen grains are connected to each other by viscin threads, and pollen is produced in eight large, elongated anthers that dehisce longitudinally (Szklanowska and Czubacki 2000; Antoń and Denisow 2018). Some species assigned to this subsection have recently been used as a pharmacological crop for the extraction of the fatty acid γ-linolenic acid from their seed (Bosisio 1990).

In addition, members of subsect. *Oenothera* ($2n = 2x = 14$), examined in the present paper, represent the best studied example to date of an uncommon and anomalous cytological phenomenon, namely permanent translocation heterozygosity (PTH; Raven 1979; Golczyk et al. 2014). The taxonomy of the genus *Oenothera* is very complicated, especially with regard to section *Oenothera* and subsection *Oenothera*, and there are at least two alternative taxonomical approaches, the first based mostly on cytogenetical studies (referred to as 'American school'; e.g., Dietrich et al. 1997), and the second, population-based concept that takes into account Renner's chromosomal complexes and 'constant' phenotypes (referred to as 'European school'). The latter is supported by Professor Krzysztof Rostański and his co-workers (e.g., Rostański 1985; Rostański et al. 2010; Rostański and Verloove 2015). According to Rostański et al. (2004), the species concept of the 'American school' is misleading as it distinguishes only 13 species of *Oenothera* within subsect. *Oenothera*, each having a long list of synonyms, which, in fact, are true species in their own right, having their own characters and distribution. The last authors also claimed that within the 'American school' concept, the broadly treated *Oenothera* species represent a mixture of quite different taxa best treated as species sensu *latissimo*. Therefore, so as to adopt a more analytical approach, we favor the concept proposed by the 'European school' for the present paper.

Flowering and flower development

The onset and duration of the flowering period were recorded for all plants investigated in 2013–2015. In order to establish the length of anthesis, times of nectar production and pollen availability, we randomly marked buds ($n = 15$; per year/per species) approx. 3 days before anthesis. Then, twice a day, i.e., in the morning (c.a. 9:00) and evening (i.e., c.a. 19:00), we noted the progress of flowering and presentation of floral rewards (i.e., pollen and nectar). Since flowers of *Oenothera* appeared to release pollen at the bud stage (i.e., approx. 24–36 h before anthesis), buds damaged during observations of pollen availability (approx. 3–5 buds, per year/per species) were excluded from further analysis of floral longevity and nectar production.

Pollen/ovule ratio and pollen quality

In order to estimate the pollen to ovule ratio (P/O; Cruden 1977), the number of pollen grains per anther per flower, as well as the number of ovules, was estimated in 2014 for a single flower per plant. The flowers ($n = 10$) were collected from different individuals ($n = 10$ individuals per species) and fixed in FAA solution (formalin: acetic acid: ethanol at a ratio of 5:5:90, by volume) in individual vials. The number of ovules was estimated for ten flowers collected from different individuals ($n = 10$ individuals per species). In determining pollen counts, only mature, undehisced anthers were used for calculations as follows. Anthers were placed in disposable centrifuge tubes, macerated and extracted in 1 ml 70% ethanol. Following vortexing, 50 µl of suspension containing pollen grains was placed on microscope slides. A coverslip was added and pollen grains scored by means of macroscopic and microscopic observations performed using an Olympus SZX12 (Tokyo, Japan) stereomicroscope and Nikon Eclipse E200 light microscope, respectively. Six replicates were made for each pollen suspension sample. Four

anthers per flower were analyzed in order to obtain the average value for a single anther. This was then multiplied by eight (the number of anthers per flower) in order to arrive at the mean number of pollen grains per flower.

The quality of pollen was assessed for 2013 and 2014 by testing for viability using a standard 2% (w/v) aqueous acetocarmine–glycerin solution (1:1) placed on microscope slides. Pollen was collected from ten flowers per species per year, and the observations were performed using a Nikon Eclipse E200 light microscope.

Stigma receptivity

The receptivity of stigmas was tested in the field using the Peroxtesmo Ko. (Merck) method (Dafni and Maues 1998). In 2013 and 2014, stigma receptivity was scored for flowers ($n = 10$ per species/per stage/per year) and three stages, i.e., (1) bud stage (2 days and 1 day, respectively, before anthesis), (2) at the onset and (3) at the end of anthesis.

Breeding system

The breeding system of the investigated populations of five *Oenothera* species was studied for the period 2013–2015. Every year, we randomly selected and marked 50 buds (per species; ca. 3 days before anthesis) on different individuals ($n = 15$); all pollination treatments were replicated within particular individuals. Also, in 2013, randomly chosen buds ($n = 10$ per species, from ten different individuals) were emasculated and enclosed within tulle isolator bags in order to test for the presence of apomixis. As a result, a total of 800 flowers were used during the course of 3 years of experiments. The flowers were divided into five groups and subjected to the following pollination treatments ($n = 10$ flowers for each treatment per year per species): (I) control—flowers left undisturbed for open pollination; (II) geitonogamy—buds (approx. 2 days before anthesis) carefully emasculated without disturbing floral development and before the anthers had dehisced, enclosed within tulle isolator bags (mesh size < 1 mm) and then hand-pollinated once the corolla had opened with pollen collected from the same individual; (III) spontaneous self-pollination—buds enclosed within tulle isolator bags to prevent insect visits; (IV) spontaneous cross-pollination—buds emasculated as above and then left uncovered; and (V) induced cross-pollination—buds emasculated as above, enclosed within tulle isolator bags and then manually pollinated with fresh pollen collected from a different individual. About 3–5 days following the end of anthesis, the flowers were unbagged and left in the experimental plots. Every year, potentially damaged flowers resulting from emasculation were excluded from further experiments. Despite the large number of flowers produced per inflorescence, it was essential for all investigated species that 3–5 flowers were open simultaneously on any given inflorescence, so as not to necessitate the emasculation of additional flowers not used in the experiments, thereby preventing uncontrolled pollen transfer from other flowers borne on the same inflorescence. Therefore, the results presented in this paper are an accurate indication of final seed production following all pollination treatments employed.

Capsule collection

Capsules were harvested 4–6 weeks following pollination and stored separately in paper envelopes until they could be analyzed. Damaged capsules, as well as ones showing signs of herbivore activity (e.g., holes or droppings), were excluded from further analyses. In the laboratory, the length of the fruit (to an accuracy of 0.1 mm) was measured. Capsules were then placed in a Petri dish, and the number of normally developed seeds calculated for each sample.

Flower visitor observations and identification

Observations of insect visitors to flowers were conducted during the peak flowering period (i.e., early July) for five separate days during 2013 and 2014 for all investigated *Oenothera* species. Owing to the specifically nocturnal anthesis of the species studied, observations began each day at approx. 19:00 and lasted throughout the night until 9:00–10:00 (GMT + 2 h). Thus, each day comprised 16 rounds of observations at 1 h intervals in the experimental area (approx. 1–2 m^2 for each species). Each round of observations was performed by two people (for each species), lasted 10–15 min and comprised: (i) observation of all insect visits to an individual plant species in order to establish the spectrum of insect visitors/frequency, (ii) observations of insect behavior, (iii) measurement of the duration of insect visits, (iv) insect photography and (v) insect capture for further analysis of pollen loads. In periods of very strong wind or rain, observations were halted and completed at the corresponding time on the days that followed. Insect visitors were assigned to five groups: Lepidoptera, *Bombus* spp., *Apis mellifera*, Coleoptera and Diptera. Insect identifications were based on the available taxonomic keys or performed by the regional specialist entomologist.

In addition, in 2014, in order to undertake detailed observations of the specific behavior of nocturnal floral insects visiting the investigated *Oenothera*, video recording was employed. This was achieved by means of a digital video camera (GZ-E10RU, JVC Everio, JVC Kenwood Corp., Japan).

Pollen load analyses

For insect pollen load analyses, we used insects captured in 2013 and 2014 visiting the five investigated species of *Oenothera*. Insect visitors were collected with an entomological net and killed in a jar containing ethyl acetate vapor. Insects were then pinned and taken back to the laboratory for further quantitative analysis of the pollen that they carried. In the case of bees (i.e., *Apis mellifera* and *Bombus* spp.), we did not analyze corbicular loads, since this pollen is not accessible during pollination. In order to quantify the pollen grains carried by each visitor, we employed the method used by Zych (2007). Pollen samples were analyzed using a Nikon Eclipse E200 (Tokyo, Japan) light microscope, and the number of pollen grains of *Oenothera* and 'other' species calculated. In the case of large pollen loads (> 500), pollen grains were calculated from nine regions consistently present on the cover slip and then extrapolated.

Statistical analyses

Data are presented as mean values ± SD (standard deviation). When possible, parametric statistical analysis was applied. Post hoc comparison of means was tested using the HSD Tukey test. The Kruskal–Wallis test was used to obtain differences for non-normally distributed data. The Spearman correlation coefficient was used to test the relationship between capsule length and seed set for each investigated species. The level of statistical significance was $P = 0.05$. All statistical analyses were performed using Statistica 13.1 (Statsoft Poland, Cracow).

Results

Flowering and floral biology

The dates of flowering for all species are shown in Table 1. In general, the onset of flowering of investigated *Oenothera* species occurred in mid- or late June, and flowering lasted until late July and/or early August. Flowers of all species exhibited nocturnal anthesis; i.e., flowers of *Oenothera* opened specifically late in the evening, i.e., 20:00–21:00, and lasted until early morning, i.e., 7:00–9:00. The flowering time of a single flower was similar for all species examined ($F_{4,142} = 2.456$, $P = 0.07$; Fig. 1) and relatively short, lasting 10.3 ± 3 h (mean ± SD, calculated across 2 study years and species). Nectar production in all *Oenothera* species commenced at the bud stage, approx. 4–5 h before anthesis. Conversely, the release of pollen from anthers began much earlier than did nectar production, i.e., approx. 24 h before anthesis (*O. flaemingina* and *O. nuda*) and approx. 36 h before anthesis (*O. casimiri, O. paradoxa, O. rubricaulis*). Significant differences in the period of nectar production ($F_{4,142} = 3.168$, $P = 0.032$) and pollen availability ($F_{4,142} = 5.343$, $P = 0.004$) were observed for all species (Fig. 1; means calculated across 2 study years).

The number of pollen grains per anther, as well as the number of pollen grains produced per flower differed significantly between species ($F_{4,32} = 15.851$, $P < 0.001$; Table 1). The greatest number of pollen grains per anther and per flower was observed for *O. flaemingina* (2848.2 ± 656.0 and 22,785.6 ± 5247.8; mean ± SD; respectively), whereas the lowest values were seen in *O. casimiri* (981.6 ± 152.8 and 7852.8 ± 1222.7, respectively). Moreover, the number of ovules per flower was also found to vary between species ($F_{4,32} = 69{,}224$, $P < 0.001$), the greatest number of ovules per flower being recorded for *O. paradoxa* (i.e., 600.6 ± 25.5)

Table 1 Dates of flowering and details of pollen number, pollen/ovule ratio and pollen viability in the five *Oenothera* species

Species	Dates of flowering			No. of pollen grains/anther	No. of pollen grains/flower	No. of ovules/flower	P/O ratio	Mean pollen viability (%)
	2013	2014	2015					
O. casimiri	14 June–25 July	15 June–06 August	18 June–29 July	981.6a ± 152.8	7852.8a ± 1222.7	313.1a ± 22.4	24.7a ± 4.7	60.5ab ± 5.2
O. flaemingina	17 June–13 August	11 June–31 July	13 June–03 August	2848.2b ± 656.0	22,785.6b ± 5247.8	405.7bc ± 41.6	57.6b ± 16.4	55.1a ± 13.9
O. nuda	18 June–30 July	12 June–07 August	17 June–27 July	1963.6c ± 149.7	15,708.8c ± 1197.5	453.3b ± 30.0	34.3a ± 3.7	65.1b ± 4.2
O. paradoxa	26 June–15 August	28 June–18 August	24 June–14 August	2215.3bc ± 721.7	17,722.7bc ± 5773.7	600.6d ± 25.5	29.6a ± 9.5	77.1c ± 7.2
O. rubricaulis	18 June–06 August	16 June–02 August	22 June–30 July	1017.0a ± 219.3	8136.0a ± 1754.5	349.6ac ± 18.8	23.4a ± 6.0	72.4c ± 3.9

Numeric data represent mean values ± SD (standard deviation). Means with the same small letter do not differ significantly among plant species at $P < 0.05$, based on the HSD Tukey test

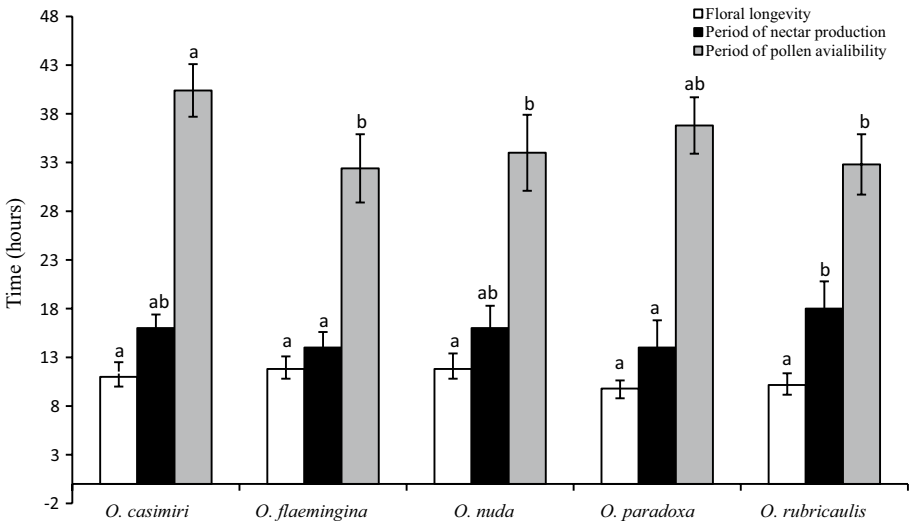

Fig. 1 Mean floral longevity, period of nectar production and pollen availability in five *Oenothera* species in Lublin, SE Poland. Data represent mean values (calculated across study years) ± SD (standard deviation). Means with the same lower case letter do not differ significantly between plant species at $P < 0.05$, based on the HSD Tukey test

and the lowest for *O. casimiri* (313.1 ± 22.4). The P/O ratio differed significantly between species ($F_{4,32} = 11.868$, $P < 0.001$), ranging from 23.4 ± 6.0 (*O. rubricaulis*) to 57.6 ± 16.4 (*O. flaemingina*). Furthermore, the viability of pollen grains ranged from 60.5 ± 5.2 to 77.1 ± 7.2% and was found also to differ between species ($F_{4,113} = 29.737$, $P < 0.001$; means calculated across 2 study years).

Field tests showed that peroxidase activity in the stigma commences prior to anthesis in all the species investigated. The stigmas were receptive at bud stage (approx. 1 day prior to anthesis) and remained receptive until the end of anthesis.

Breeding system

From the 800 flowers of all the species used experimentally, we collected 207, 211 and 169 capsules in 2013, 2014 and 2015, respectively. The remaining capsules had been partly consumed/destroyed by herbivores (for the analyses, we used only undamaged capsules) or could not be found during capsule harvesting. With the exception of those flowers where anthers had been removed and flowers bagged from bud stage and throughout anthesis (test for apomixis), capsules formed by flowers subjected to all pollination treatments contained seed. In general, all species differed in the number of seeds developed per capsule, subject to the different pollination treatments employed (Fig. 2; Table 2). Nevertheless, no common pattern was observed between species, and differences discovered were not always statistically significant. On the one hand, the lowest seed set was observed for emasculated and spontaneously cross-pollinated flowers (treatment IV) of *O. flaemingina* (151.8 ± 39.8; 37.4% of ovules), *O. nuda* (152.4 ± 37.5; 33.3% of ovules), *O. paradoxa* (252.5 ± 114.6; 42.0% of ovules), *O. rubricaulis* (137.6 ± 63.3; 39.3% of ovules) and in induced cross-pollinated flowers (treatment V) of *O. casimiri* (177.3 ± 83.6; 56.6% of ovules; data calculated over the 3 years period). On the other, the greatest number of seed sets occurred in open-pollinated control flowers (treatment I) of *O. flaemingina* (210.3 ± 26.7; 51.8% of ovules), *O. nuda* (205.1 ± 48.4; 45.2% of ovules), *O. paradoxa* (366.4 ± 92.9; 61.1% of ovules) and geitonogamously pollinated flowers (treatment II) of *O. casimiri* (264.6 ± 62.0; 84.5% of ovules) and *O. rubricaulis* (229.6 ± 39.3; 65.7% of ovules; data calculated over the 3 year period). In all species, intact control flowers produced as many seeds as hand-cross-pollinated flowers (Fig. 2). Considerable variation in average seed set in all *Oenothera* species was recorded between the 3 years of study for any given pollination treatment (Fig. 2), with more seed set observed for each species in 2015. Furthermore, the pollination treatment × year interaction was also significant for all species (Table 2).

Capsule length was significantly correlated with seed set for *O. casimiri* (Spearman $r_s = 0.69$), *O. flaemingina* ($r_s = 0.66$), *O. nuda* ($r_s = 0.78$), *O. paradoxa* ($r_s = 0.71$) and *O. rubricaulis* ($r_s = 0.82$; $P < 0.001$). The longest capsules were produced in control open-pollinated flowers (treatment I) of *O. casimiri* (23.4 ± 4.1 mm), *O. flaemingina* (25.9 ± 1.9 mm), *O. paradoxa* (32.2 ± 2.2 mm) and in cases of geitonogamous pollination (treatment II) in *O. nuda* (26.1 ± 3.4 mm) and *O. rubricaulis* (23.6 ± 1.5 mm). The shortest capsules, on the other hand, occurred in spontaneously cross-pollinated flowers (treatment IV) of *O. paradoxa* (25.7 ± 4.3 mm), *O. rubricaulis* (20.2 ± 1.6 mm), *O. nuda* (22.3 ± 1.3 mm) and in induced cross-pollinated flowers (treatment V) of *O. casimiri* (20.4 ± 1.6 mm) and *O. flaemingina* (22.7 ± 0.9 mm; $P < 0.05$; means calculated across 3 study years).

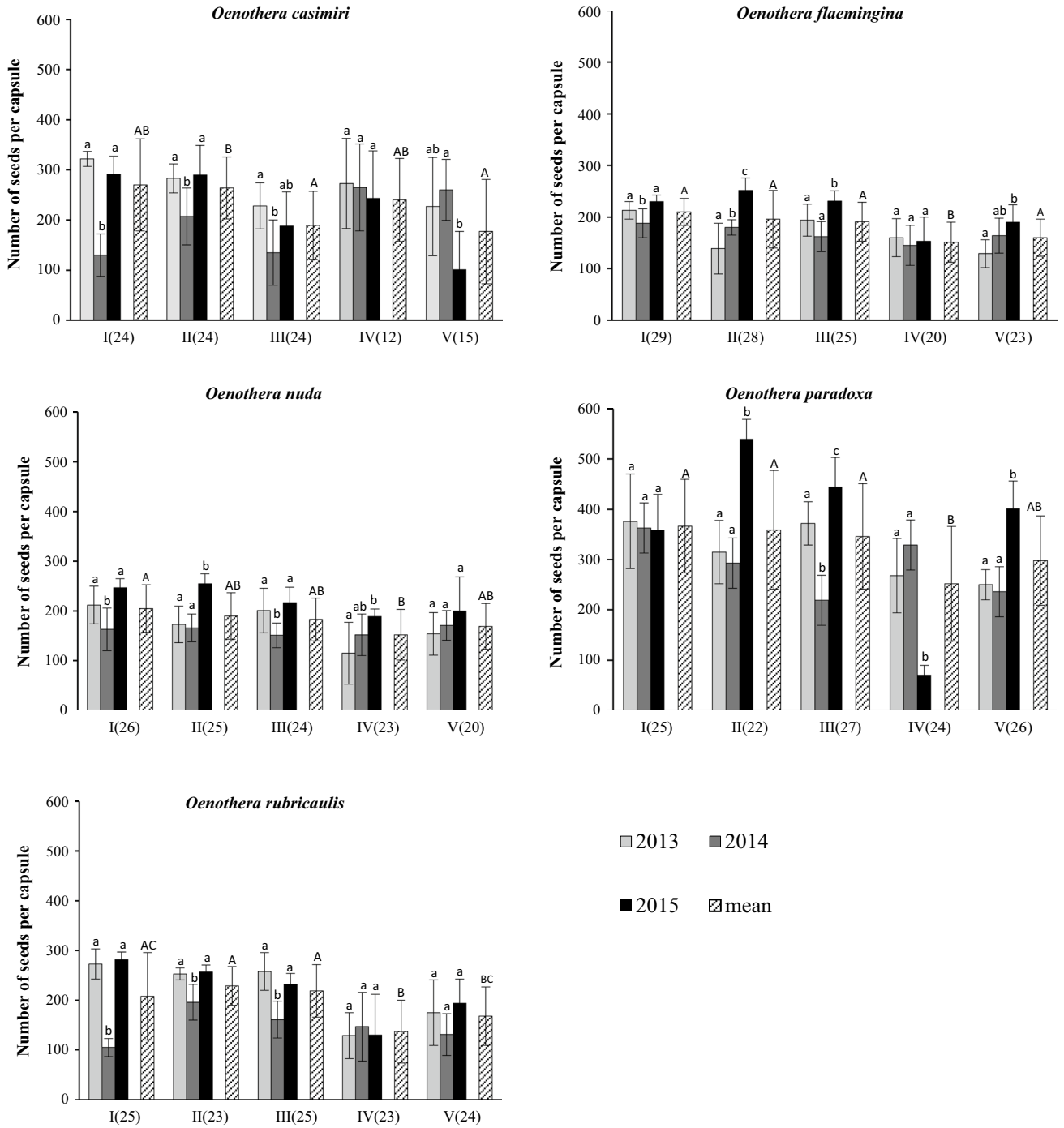

Fig. 2 Average seed set ± SD (standard deviation) in five *Oenothera* species subjected to five pollination treatments in 2013–2015: (I) open pollination (control); (II) buds emasculated before the anthers dehisced, then hand-pollinated with pollen collected from the same individual (geitonogamy); (III) spontaneous self-pollination; (IV) spontaneous cross-pollination; and (V) induced cross-pollination. The numbers in brackets by the pollination treatment symbol indicate the number of harvested capsules. Means with the same lower case letter do not differ significantly between study years for a particular pollination treatment, whereas means with the same capital letter do not differ significantly between pollination treatments employed, at $P < 0.05$, based on the HSH Tukey test

Table 2 Results of two-way model analysis of variance (ANOVA) on number of seeds per capsule in five *Oenothera* species

Species	Variable	*df*	*N*	Sum of squares	Mean square	*F*	*P*
O. casimiri	Year	2	96	98,413.60	49,206.80	7.293	0.002
	Treatment	4	94	120,933.60	30,233.39	4.546	0.003
	Year × treatment	14	84	436,035.4	31,145.39	8.437	< 0.001
O. flaemingina	Year	2	122	50,844.05	25,422.02	14.622	< 0.001
	Treatment	4	120	61,710.02	15,427.50	9.199	< 0.001
	Year × treatment	14	110	156,561.00	11,182.93	11.562	< 0.001
O. nuda	Year	2	115	75,557.18	37,778.59	19.634	< 0.001
	Treatment	4	113	39,160.17	9790.60	4.293	0.003
	Year × treatment	14	103	146,796.90	150,033.90	7.198	< 0.001
O. paradoxa	Year	2	122	143,034.20	71,517.09	6.164	0.003
	Treatment	4	120	218,175.10	54,543.77	4.883	0.001
	Year × treatment	14	110	105,295.50	50,593.60	16.363	< 0.001
O. rubricaulis	Year	2	117	141,457.40	70,728.72	18.033	< 0.001
	Treatment	4	115	139,630.20	34,907.55	8.713	< 0.001
	Year × treatment	14	105	396,044.60	28,288.90	14.538	< 0.001

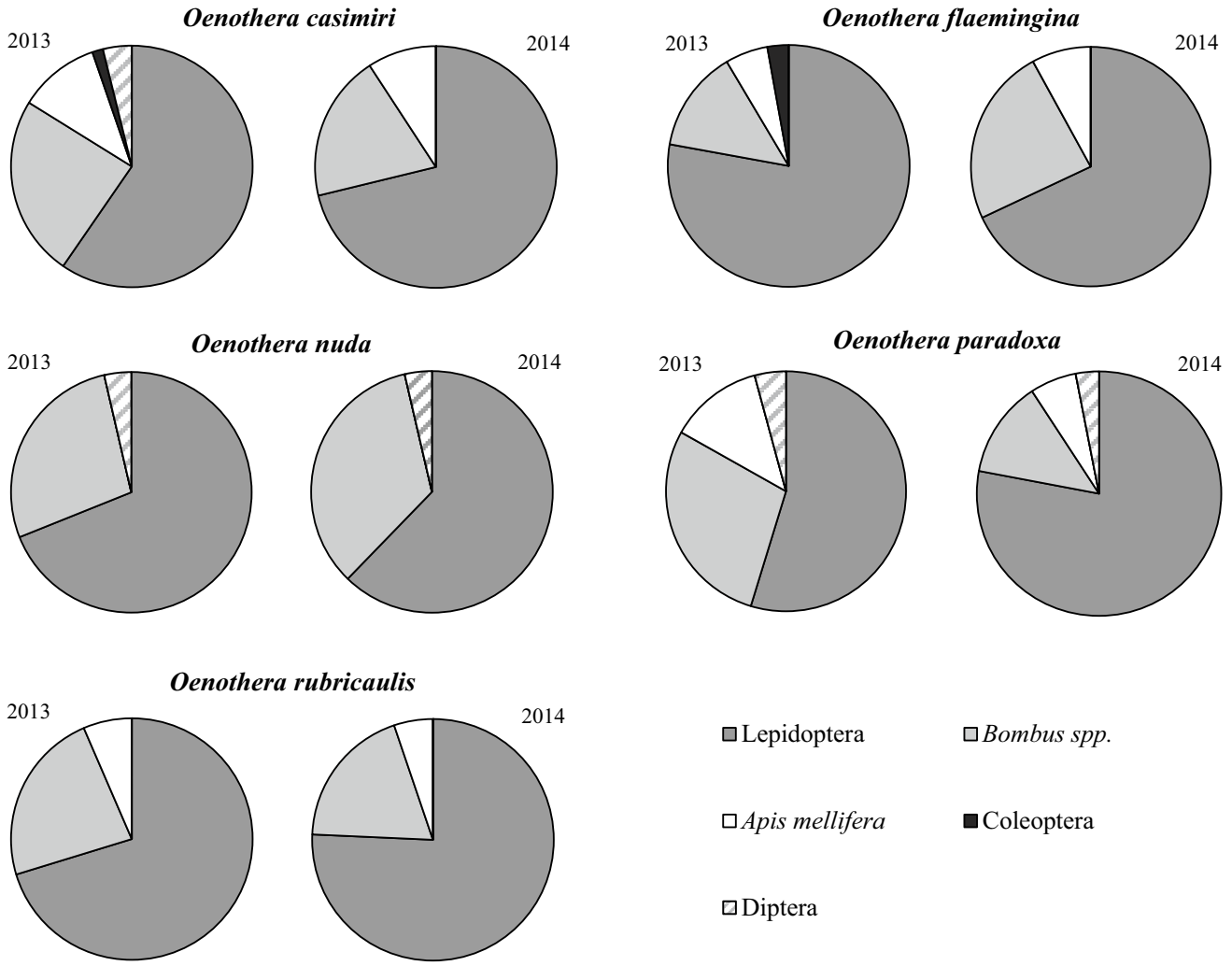

Fig. 3 Composition (%) of the floral insect visitors in five *Oenothera* species in a Lublin, SE Poland population, based on observations in 2013 and 2014

Floral insect visitors

During each of the study seasons, both nocturnal and diurnal insects were seen visiting flowers of *Oenothera.* They belonged to four taxonomic orders, namely Coleoptera (Scarabaeidae: *Oxythyrea funesta* Poda); Diptera (Syrphidae: *Episyrphus* sp. Matsumura and Adachi); Hymenoptera (Apidae: *Apis mellifera* L., *Bombus hortorum* L., *B. lapidarius* L., *B. pascuorum* Scopoli, *B. pratorum* L., *B. terrestris* L.); and Lepidoptera (Noctuidae: *Autographa gamma* L., *Pyrrhia umbra* Hufnagel). Insects from orders Hymenoptera and Lepidoptera were observed in both 2013 and 2014 visiting all *Oenothera* species (Fig. 3). Conversely, species of fly (Diptera) were observed visiting only *O. casimiri, O. nuda* and *O. pardoxa*, whereas species of beetle (Coleoptera) visited only *O. casimiri* and *O. flaemingina* in 2013. Given that the last two insect taxa were observed only very rarely and represented no more than 4% of the total insect visits, we decided to exclude them from further analyses. Moreover, since flowers of *Oenothera* present pollinators with no morphological restrictions, we refer, hereafter, not to a particular insect species but to morphologically related (or functional sensu Fenster et al. 2004) groups of insects, i.e., Lepidoptera, honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.).

For all study seasons, most floral visits to *Oenothera* were made by nocturnal Lepidoptera. Indeed, the proportion of visits made by lepidopterans to any individual *Oenothera* species much exceeded 50% of all insect visits in both 2013 and 2014 (Fig. 3). Nocturnal Lepidoptera began visiting flowers of *Oenothera* prior to dusk, just after the first flowers opened, and the greatest activity of foraging was observed late in the evening (i.e., 21:00–23:00 GMT + 2 h). During visits to flowers, nocturnal Lepidoptera displayed specific foraging behavior; i.e., these insects landed exclusively at the edges of petals and consumed floral nectar from the hypanthium using their long proboscises. Remarkably, these insects were observed only extremely rarely to come into contact even with just one of the eight anthers or stigmatic lobes (Online Resource 1).

Bumblebees were the most frequently observed diurnal floral insect visitors to *Oenothera*, in both 2013 and 2014, representing 25.2% and 19.6 for *O. casimiri*, 13.9% and 24.0% for *O. flaemingina*, 28.1% and 35.9% for *O. nuda*, 27.0% and 12.5% for *O. paradoxa* and 24.7% and 19.1% for *O. rubricaulis*, respectively, for 2013 and 2014 (Fig. 3). Honeybees were not observed visiting *O. nuda* in any of the study years, whereas in other *Oenothera* species, honeybees were recorded relatively rarely, totalling some 5.2–10.5% of all insect visits (mean data for all plant species). Both bumblebees and honeybees began foraging early in the morning (i.e., approx. 5:00) and visited flowers until the onset of senescence (i.e., approx. 7:00–9:00), consuming both pollen and floral nectar.

Duration of insect visits and pollen loads analyses

Given that the species of *Oenothera* investigated in this paper do not vary significantly in the diversity or abundance of insects attracted to their flowers, we decided to pool the pollen load data and duration of insect visit data for the entire study period and for all plant species.

On average, the duration of insect visits to *Oenothera* flowers was 6.9 ± 4.1 s. However, the duration of visits differed considerably between groups of insects (Kruskal–Wallis test: H (2, N = 149) = 19.4348, $P < 0.001$). The longest visit to *Oenothera* was recorded for honeybees (i.e., 7.9 ± 3.2 s), whereas the shortest visit was observed for bumblebees (i.e., 5.1 ± 1.5 s).

In general, all captured insect visitors carried pollen of *Oenothera* and/or 'other' plant species. However, there were considerable differences in the number of pollen grains carried by the various insect groups, of both *Oenothera* pollen (Kruskal–Wallis test: H (2, N = 86) = 57.820, $P < 0.001$) and 'other' pollen (Kruskal–Wallis test: H (2, N = 86) = 25.383, $P < 0.001$). Although nocturnal Lepidoptera was the group of insects most frequently observed visiting flowers of *Oenothera* (Fig. 3), pollen load analyses revealed that these insects carried only very small numbers of pollen grains, of both *Oenothera* and 'other' species, on their bodies (i.e., 4.46 ± 4.32 and 22.50 ± 11.47, on average, respectively; Table 3). The greatest number of pollen grains of *Oenothera* was carried by honeybees (on average, 376.40 ± 355.31) and bumblebees (on average, 313.62 ± 211.28), and there was no significant difference between these groups of insect for *Oenothera* pollen loads (Kruskal–Wallis test: H (1, N = 56) = 0.198, $P = 0.888$). The greatest *Oenothera* pollen load for *A. mellifera* was 1740 pollen grains, whereas that for *Bombus* spp. was 685 pollen grains.

Table 3 Average number of pollen grains of *Oenothera* and 'other' plant species carried by the most frequent group of floral insect visitors (Diptera and Coleoptera excluded)

Insect group	*n*	*Oenothera* pollen grains	'Other' pollen grains
Lepidoptera	30	4.46 ± 4.32	22.50 ± 11.47
Apis mellifera	24	376.40 ± 355.31	101.87 ± 186.64
Bombus spp.	32	313.62 ± 211.28	75.62 ± 76.58

Data represent mean values (calculated from all study seasons and species) ± SD (standard deviation). Significant differences were found for *Oenothera* pollen loads and 'other' pollen loads carried among particular insect groups; Kruskal–Wallis test, $P < 0.001$)

Discussion

Our results clearly indicate that all investigated *Oenothera* are self-compatible and reproduce sexually. Moreover, flowers emasculated and bagged throughout anthesis did not produce any seed, and thus, apomixis can be dismissed. Data from field investigations also revealed that the receptivity of the stigma and the release of pollen grains from anthers began at bud stage (approx. 24 h before anthesis), and the stigmas remained receptive until the onset of flower senescence. As a result of these developmental and temporal overlaps of male and female functions within flowers of *Oenothera*, self-pollination at the final bud stage (just before the flowers opened) was frequently observed. This phenomenon of self-pollination has been termed 'prior selfing' (or 'bud autogamy' sensu Noormets and Olson 2006, or 'preanthesis cleistogamy' sensu Culley and Klooster 2007) and has only rarely been recorded in self-fertilizing species (but see, e.g., Davis and Delph 2005; Bush 2009; Ling et al. 2017). We also demonstrated that bagged, intact flowers produced as many seeds as flowers subjected to hand-pollination treatments. This finding strongly indicates that the investigated species of *Oenothera* have a great capacity for autonomous selfing, which is consistent with the findings for other breeding system experiments on several members of subsect. *Oenothera* (Stubbe and Raven 1979; Kawano et al. 1995). Nevertheless, the pattern observed here does not apply to the whole genus, since both self-compatible and self-incompatible populations as well as variable populations have been reported for other sections and subsections of *Oenothera* (e.g., Gregory 1964; Wagner and Lammers 2005; Theiss et al. 2010).

In general, selection in favor of autonomous selfing is influenced by the degree of pollination variability, and selfing is particularly advantageous when the frequency of pollinator visits and/the reception of outcross pollen are low, as it offers reproductive assurance (Darwin 1876; Baker 1955; Goodwillie et al. 2005). According to Cruden (1977), analysis of the P/O ratio is a valuable indicator of breeding systems in flowering plants, and therefore, outcrossing rates within a species. This last author also suggested that in primarily self-pollinating taxa, investment in male fitness is expected to be lower compared to female fitness, and thus, the more efficient the transfer of pollen, the lower the P/O ratio. We observed that the P/O ratio of *Oenothera* was relatively low (33.9 ± 13.3, on average) and varied between species. Even so, the modified P/O ratio generally agreed well with that of other *Oenothera* spp., including the well-studied and closely related *O. biennis* and *O. flava* (Cruden and Jensen 1979; Summers et al. 2015). In Onagraceae, the reproductive structures are conspicuous because pollen grains are held by viscin threads and the stigmas are relatively large. Consequently, these characteristics probably contribute to the relatively low *P*/*O* ratio (Cruden and Jensen 1979). We thus propose that in the case of the *Oenothera* species investigated, a low P/O ratio is more closely associated with efficient pollen transfer onto the body of the pollinator than with shifts in outcrossing rates. Therefore, despite relatively low *P*/*O* ratios, it is proposed that large clumps of pollen coupled with the large surface area of the stigma contribute to successful pollination in these species. As such, it is also worth noting that significant variations in P/O ratio have also been demonstrated for several populations of *O. flava* in North America, with respect to geographical or temporal variability in pollinator abundance and mating opportunity (Summers et al. 2015).

Our study shows that flowers of the five *Oenothera* species, typically classified as 'obligately autogamous' (sensu Cruden 1977), may be fertilized either by autonomous (e.g., via prior selfing) or outcross pollen (after flower opening), indicating the potential for a mixed mating system (sensu Goodwillie et al. 2005). As a result, these plants, to different degrees, are subject to the dual advantages of both selfing and outcrossing. For example, autonomous selfing can precipitate pollination even when marked spatiotemporal variation in pollinator service is encountered, or mates are scarce. Indeed, we were able to demonstrate that emasculated flowers exposed to pollinators set fewer seeds than intact control flowers subject to open pollination; this difference, however, was not statistically significant for *O. casimiri*. This finding strongly indicates that for the species of *Oenothera* investigated here, selfing contributes appreciably to reproductive assurance (sensu Lloyd 1992). In addition, given that pollen availability and stigma receptivity were observed until the onset of flower senescence, it is proposed that competing and/or delayed selfing (either autonomous or vector mediated) may also occur during more advanced stages of floral development. A similar phenomenon is also thought to occur in the flowers of *Tillandsia multicaulis* Steud. (Bromeliaceae; Bush 2009) and *Prunella vulgaris* L. (Lamiaceae; Ling et al. 2017), in which self-fertilization was observed prior to flower opening, but seed production increased with progressing anthesis. In reality, however, the timing of autonomous self-pollination does not always fit into the three strict categories outlined here as it is continuously distributed in time, and therefore, selfing is always associated with some costs to both pollen and seed discounting (Lloyd 1992).

The breeding experiments were performed for only 3 years; however, we noted great variations in the total seed set between study years for most pollination treatments and all plant species. According to Nayor (2003), the number of seed produced is a phenotypic character that reflects the

interaction between the genetic potential to produce seed and the environment experienced by the individual plant. This last author also suggested that environmental conditions during flowering and seed development may affect the proportion of fertilized ovules which are retained and the amount of reserves that they accumulate. Given that the anthesis of a single flower of the investigated species of *Oenothera* is relatively short (ca. 10-12 h), it is reasonable to suppose that ever-changing weather conditions (e.g., temperature, air humidity) may considerably impact on pollination and fertilization in these taxa. Indeed, it has been demonstrated that immediate and small-scale weather effects may act as constraints on the survival and germination of pollen grains, the exposure and receptivity of the stigmas, as well as pollinator activity in the field (e.g., Corbet 1990).

Although a substantial amount of autogamy appears to occur in the investigated *Oenothera*, a certain level of outcrossing is also needed in order to produce higher quality offspring and to create new PTH forms (Golczyk et al. 2014). We observed that flowers of *Oenothera* were visited by a great variety of potential pollinators, including both diurnal and nocturnal taxa. Remarkably, hawkmoths were not observed during our investigations for any study years, even though our experiments were conducted in a botanical garden likely to exhibit high insect biodiversity. It is generally agreed that the great requirement of pollinators for energy necessitates the uptake of much nectar (Nicolson 2007 and references therein). In the case of the species of *Oenothera* studied, insect visitors were attracted to the flower by both copious amounts of floral nectar (i.e., up to 26.8 mg/flower; Antoń et al. 2017) and pollen (i.e., up to 6.4 mg/flower; Antoń and Denisow 2018) that were constantly accessible from the onset of anthesis (i.e., evening hours) until the start of flower senescence (i.e., early morning hours). It is also worth noting that it has been proposed for other species of subsect. *Oenothera* that both visual (strong UV-absorbent spots near the center of the corolla) and chemical (volatiles with linalool as a primary constituent) signals participate in the attraction of potential pollinators to flowers (Kawano et al. 1995); however, these signals were not examined in our investigations. Even though, for every year of study, nocturnal lepidopterans were the most frequently encountered floral insect visitors (much exceeding 50% of all insect visits; see Fig. 3), our observations clearly show that these insects did not in any way contribute to the pollination of *Oenothera*. This was probably due to the specific behavior of these nocturnal lepidopterans during foraging, which differs significantly from that observed for hawkmoths. Indeed, it was demonstrated that during relatively long floral visits, these insects eagerly consumed floral nectar. Even so, they did not come into contact either with the anthers or with the lobes of the stigma (see Suppl. mat. 1). The findings were further confirmed during pollen load analyses, in that these insects carried extremely low numbers of *Oenothera* pollen grains on their bodies. Conversely, however, hawkmoth visits comprise a series of successive phases, including repeated backward and downward movements, hovering near the flower, the proboscis frequently entering the spur or nectar tube and thereby increasing the frequency of pollination events (Brantjes and Bos 1980). Consequently, given that during visits nocturnal lepidopterans did not come into direct contact with the reproductive parts of the flower, they can be considered 'nectar thieves' (sensu Inouye 1980), which, owing to the absence of visible damage to floral tissues indicative of typical nectar theft, here, seemingly have a negligible effect on plant reproduction. However, the direct effect of nectar theft by these insects on plant fitness was not examined in the present paper, and this phenomenon still requires further experimental testing.

Our observations showed that the true pollinators of *Oenothera* are probably members of family Apidae (i.e., honeybees and bumblebees), even though these insects contributed to significantly fewer visits and never exceeded more than 12% and 33% of total insect visits, respectively. Fidelity of these insects to *Oenothera* flowers is obvious from pollen load analyses. Indeed, in this study we demonstrated that generally, most honeybee and bumblebee pollen loads consisted mainly of *Oenothera* pollen grains (78.7% and 80.6%, respectively; Table 3). According to Dietrich et al. (1997), in chiefly self-pollinating *Oenothera*, floral rewards and visits by insects are less important. More recently, however, Ragusso et al. (2007) have shown that the high volumes of nectar present in small-flowered *O. triloba* (formerly referred to as autogamous) may indicate the potential for a mixed mating system, rather than strict autogamy. Our findings are consistent with those of the latter authors and demonstrate that, apart from prior selfing, visits by bees may lead to effective transfer of outcross pollen in the investigated species of *Oenothera*. Furthermore, in another, complementary project (Antoń et al. 2017), we reported that the complex pattern of nectar production and nectar distribution along the inflorescences of these taxa may determine the movement of pollinators between individual plants, thereby promoting cross-pollination. These observations all indicate that the breeding system present in subsect. *Oenothera* is much more intricate than previously thought, and therefore, these species should not be simply considered autogamous. Additionally, and in contrast to previous studies, which describe *Oenothera* plants as having generalized pollination systems (e.g., Waser et al. 1996), Krakos and Fabricant (2014) demonstrated that pollination systems in several *Oenothera* appear to be more pollinator specialized. The latter results, however, are limited only to outcrossing species from section *Gaura*, occurring in Northeast, Midwest, and Southwest of the USA.

It is also interesting to speculate on certain aspects of *Oenothera* pollination and breeding system strategies and their involvement in the dynamic spread of these taxa and their colonization of new localities. According to Tokhtar and Groshenko (2014), the representatives of subsect. *Oenothera* have become successfully established in Europe, and some taxa are considered to be actively invasive species, especially in Central and Eastern Europe. Invasion by alien plant species is recognized to be a serious threat to global biodiversity. However, despite growing concern over the negative aspects of plant invasion on various levels, surprisingly little is known about those attributes that make these plants such successful invaders (Richardson et al. 2000). The capacity of members of subsect. *Oenothera* to invade has been explained in terms of the similar weather conditions that prevail in Central Europe and which well match the climate present in their primary distribution range (Mihulka and Pyšek 2001), and/or their requirement for light in the initiation of seed germination (Mihulka et al. 2003). Here, we show that the potential of five species of subsect. *Oenothera* to invade is probably improved by a high degree of selfing (occurring inter alia at bud stage), followed by anthesis and opportunities for outcrossing, as well as the hybridization of new PTH forms. These characteristics are frequently associated with the invasive spread of species, especially within anthropogenic habitats (Denisow et al. 2017). We conclude that the species of *Oenothera* investigated in this paper are able to ensure autonomous reproductive advantage, especially when pollinators are scarce. These strategies probably enable a rapid increase in populations, as well as the vigorous establishment of colonies in new environments outside their primary distribution range, sites that are devoid of co-adapted fauna and flora.

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Information on Electronic Supplementary Materials

Online resource 1. *Autographa gamma* during visitation in *Oenothera* flowers.

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