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ORIGINAL ARTICLE

Termination of diapause in the lime seed bug *Oxycarenus lavaterae* (Heteroptera: Lygaeoidea: Oxycarenidae)*

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Abstract. The true bug *Oxycarenus lavaterae* has spread northwards through Europe from the Mediterranean at the beginning of the 21st century. Temperature and photoperiod in particular countries are important for the prediction of further spread. The timing and conditions for the termination of winter diapause in Bulgarian and Czech populations of *O. lavaterae* were determined. The progress in diapause development in the Bulgarian population was monitored by changes in the duration of the pre-oviposition period under long day (18L : 6D) conditions, which decreased gradually from October to June from 98 to 10 days at 20°C and 62 to 9 days at 25°C. There was no change in the photoperiodic response around the winter solstice in December/January, as is recorded for many other temperate insects. Instead, most overwintering females of *O. lavaterae* transferred to the diapause-promoting short-day photoperiod (12L : 12D) at 25°C did not start to oviposit even though they lived up to one year. The percentage of females that oviposited under short day conditions gradually increased from December to May, but only reached approximately 50% at most. In the Czech Republic, the number of individuals observed resting in overwintering aggregations on tree trunks gradually decreased up to early June. This bug seems remain in diapause until lime trees flower and their seeds become available.

INTRODUCTION

Spread of non-native species of Heteroptera

Between 2000 and 2020, several species of bugs, including agricultural pests, spread mostly northwards on several continents including Europe. The brown marmorated stink bug, *Halyomorpha halys* Stål (Heteroptera: Pentatomidae), became an invasive species in Europe, parts of Asia and America (Musolin et al., 2022). Locally it causes serious economic loss to crops and when overwintering is a public nuisance (Nixon et al., 2022). The southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae), has spread into many parts of the world. Its reproductive winter diapause is controlled by photoperiod (Musolin, 2012). These species seem to have spread northwards due to climate change (Musolin, 2007; Chartois et al., 2021). In the north, they are subject to different photoperiodic regimes, which are important for diapause induction and sometimes for its termination (Musolin et al., 2019). Another non-native species of Heteroptera that is spreading northwards is the lime seed bug *Oxycarenus lavaterae* (Fabricius, 1787) (Heteroptera: Lygaeoidea: Oxycarenidae), which is the subject of this study.

Diapause in species of Heteroptera

One of the most important life-history parameters of a temperate insect that limits its ability to increase its distribution is voltinism determined by diapause regulation (Hodek, 1983, 2012). Several reviews focus on diapause in Heteroptera that mostly overwinter as adults (Numata, 2004; Saulich & Musolin, 2009, 2012; Lundgren, 2011; Musolin & Saulich, 2018). Most of these papers deal only with diapause induction. In some pentatomids (e.g. in *Palomena* spp., *Eurygaster* spp.), virtually every individual in each generation enter diapause regardless of environmental factors (they are obligatory univoltine). However, most species have a facultative diapause, the onset of which is

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** Deceased.

regulated by environmental factors (mainly photoperiod, temperature and availability of food) (Musolin & Saulich, 2018).

Diapause termination is less studied in Heteroptera. To distinguish between the multiple physiological pathways for diapause completion, Hodek (1977, 2002) defined horotelic diapause development (natural slow process occurring in winter when photoperiod is short and temperature low) and tachytelic diapause development (reactivation = an accelerated process following exposure to photoperiod and temperature similar to that occurring in summer). The horotelic diapause development was studied in *Pyrrhocoris apterus* (L.) (Heteroptera: Pyrrhocoridae). Under a constant 20°C and short-day photoperiod, the intensity of diapause decreases to medium level. Full diapause termination is stimulated by exposure to cold temperatures (Košťál et al., 2008). A very short pre-oviposition period of only several days in *Anthocoris nemorum* (L.) (Heteroptera: Anthocoridae) transferred in January from outdoors to a laboratory indicates that it is no longer in diapause (Collyer, 1967; Herard & Chen, 1985; see also comments in Saulich & Musolin, 2009). Tachytelic termination of diapause in *Riptortus clavatus* (Thunberg, 1783) (Heteroptera: Alydidae) has critical photoperiod similar to diapause induction (between 13 and 14 h) (Numata & Hidaka, 1982). Diapause termination in *Orius insidiosus* (Say, 1832) (Heteroptera: Anthocoridae) is quickly terminated when photoperiod exceeds 12L : 12D, whereas *Orius majusculus* (Reuter, 1879) remains in diapause after two weeks under a long day 16L : 8D conditions (van den Meiracker, 1994).

Biology of *Oxycarenus lavaterae*

Since 1990, the lime seed bug *O. lavaterae*, which was originally distributed in the warm Palaearctic region including the Mediterranean (Pericart, 2001), has spread northwards. Large aggregations of *O. lavaterae* were reported for the first time in Bulgaria in the winter of 1998/1999 on linden trees (Kalushkov, 2000). In the Czech Republic, it was first recorded in autumn 2004 (Kment et al., 2010). This species is considered a forestry pest (Velimirovic et al., 1992) or public nuisance (Wermelinger et al., 2005) in some countries.

Oxycarenus lavaterae develops on various Malvaceae species in the Western Mediterranean, while in Italy, central Europe and the Balkan Peninsula it occurs on linden trees (*Tilia* spp.) (Kalushkov & Nedvěd, 2010; Kment et al., 2010). In Bulgaria, the bugs gradually leave their hibernacula from March to April, start to feed and reproduce. Adults of the first generation occur in mid-June, the second in late July and third in late September, and then overwinter in diapause (Kalushkov, 2000).

These bugs are easy to rear in the laboratory; the longevity at 20°C is 63 to 113 days and fecundity 250 to 390 eggs, depending on the time spent in outdoor storage prior to termination of diapause. Mating frequency is 36% and positively correlated with fecundity (Nedvěd et al., 2014).

The objective of this study was to determine the timing and prerequisites for termination of winter diapause in the lime seed bug, *O. lavaterae*, which might limit its colonisation of geographical areas that differ in terms of temperature and photoperiod, and the number of generations produced per year.

MATERIAL AND METHODS

Laboratory experiments

The bugs used in the experiments were collected near Sofia, Bulgaria (42°40′48″N, 23°24′52″E; 530 m a.s.l.), where the longest daylength is about 17 h at the summer solstice, and about 13 h including civil twilight at the autumn equinox. The photoperiods used in the experiments: short day (SD) = 12L : 12D and long day (LD) = 18L : 6D, resemble the photoperiodic conditions *O. lavaterae* experience in the field and enable the results to be compared with those of another model insect, *P. apterus*, with a very similar life history (Hodek, 1971a, 1988).

In the first experiment, the pre-oviposition period and longevity of diapausing bugs collected in the field in November 1999 after transfer to the laboratory were recorded. The first batch of bugs were not exposed to low temperatures, and they were directly placed in the laboratory at 25°C under both SD and LD conditions. The second batch of bugs was kept for 10 weeks at 10°C before transfer to 25°C and SD.

Bugs for the second experiment were collected from trees where they overwintered in early October 2005 and kept outdoors in several 500 ml glass jars covered with nylon net and provided with zigzag folded filter paper. Linden (*Tilia cordata*) seeds with their coats removed were strewn on the bottoms of the jars as a source of food; water in glass vials with cotton plugs were exchanged once a week. Random samples of the bugs were taken each month from mid-October 2005 through to mid-June 2006 and transferred to the laboratory and kept under a LD photoperiod and constant temperatures of 20 ± 2°C and 25 ± 2°C and under a SD photoperiod at 25°C ± 2°C. Light intensity, which can affect the duration of the photophase experienced (Saunders, 2022), was about 500 lx.

Pairs of bugs were kept separately in Petri dishes (8.5 × 1.2 cm) with zigzag folded filter paper. Each pair was provided with about 10 linden seeds, along with water in a glass vial. Presence of eggs and survival of males and females were checked each day, date of the first oviposition and death of females were recorded, dead males were replaced. Percentage of egg laying females reactivated from diapause and duration of pre-oviposition period were recorded as they are widely used criteria for determining type of termination of diapause and its intensity, respectively (Tauber et al., 1986; Danks, 1987), the number of eggs was counted, and mean lifetime fecundity of the females that oviposit calculated.

The conditions for diapause induction were determined in the third experiment in which the first generation of bugs were the offspring of those collected in March 2015 in the Czech Republic (see below). Larvae were divided between SD and LD conditions and reared to the adult stage. Fifteen pairs of bugs from each photoperiod were placed in Petri dishes and reared as above for 42 days. Egg laying was checked weekly.

Field observation

Aggregations of bugs in diapause on the trunks of linden trees (*Tilia* spp.) were observed in South Bohemia, Czech Republic (48°58′44″N, 14°28′00″E; 390 m a.s.l.) in several winters. In spring 2015, the sizes of the clusters of bugs (horizontal × vertical dimension) were repeatedly measured on three trees at about 2 m above the ground.

Statistical analysis

The duration of the pre-oviposition period was analysed using a one-way ANOVA for each combination of temperature and photoperiod. Values for each month of transfer were subsequently compared using the Fisher LSD post hoc comparison for LD conditions and unequal N HSD post hoc comparison for SD conditions. Longevity at SD was analysed using ANOVA and Fisher LSD test, because the numbers of individuals in each experiment were the same each month, whereas fecundity was analysed using ANOVA and unequal N HSD test, because the numbers of females that started to oviposit differed between months.

Differences in the duration of the pre-oviposition period at the two temperatures and under LD were analysed using a t-test for each month. Because there were nine parallel comparisons (= months), the level of significance of the differences between the two temperatures in the same month were adjusted using the Bonferroni procedure (Table 1).

RESULTS

Diapause induction

Adults in the October samples used in the second experiment, which probably moulted from larvae in September, i.e. when the photoperiod was about 13.5L : 10.5D, did not reproduce. The third experiment testing conditions for diapause induction resulted in eight of 15 pairs laying eggs under LD compared to one of 15 under SD. That SD conditions are unsuitable for normal development was also indicated by the low lifetime fecundity (40–70 eggs) of the post-diapause parents in comparison with that recorded for suitable LD conditions (276 ± 150 eggs).

Effect of exposure to cold on diapause termination

In the first experiment the 10-week exposure to cold (10°C) resulted in the activation of diapausing females. Two of 10 cold-treated females laid eggs after 20 and 31 days after transfer to SD, 25°C. Only four of the 44 females that were not exposed to cold laid eggs and only after a much longer pre-oviposition period (125–252 days) under SD at 25°C. Mean (± S.D.) longevity of the 40 SD females that did not oviposit was 155 ± 74 days (median 128, range 64–377 days), whereas that of the four that did oviposit lived for 170–293 days. For those kept under LD conditions, 27 of 42 (64%) females did oviposit, with a pre-oviposition period of 52 ± 15 days (median 54, range 25–82 days). Their mean longevity was 113 ± 66 days (median 93, range 57–378 days), whereas that of those that did not oviposit was shorter, 82 ± 28 days (median 71, range 57–144 days).

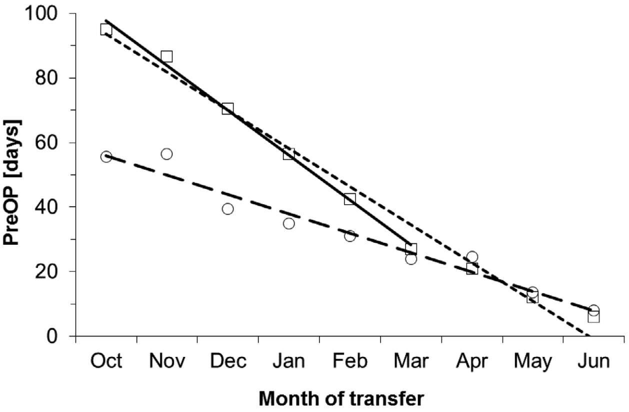

Fig. 1. Intensity of diapause of female *Oxycarenus lavaterae* in terms of their pre-oviposition period (PreOP, medians) when transferred from outdoors into long day conditions (18L : 6D) at 20°C (□ — and ···) and 25°C (○ ---). Dotted line represents the decrease in PreOP if it were gradual over the entire period (0.39 ± 0.03 days per day), solid line the decrease until the break point in March (0.46 ± 0.03 days per day), which continues to decrease from March to June at a rate of 0.24 ± 0.05 days per day, which is similar to rate at 25°C = dashed line (0.20 ± 0.01 days per day).

Termination of diapause under a long photoperiod

In the second, long term experiment, the percentage of females that oviposit was almost constant (about 90%) under LD photoperiod at both temperatures (20 and 25°C), and the duration of pre-oviposition period gradually decreased from October to June (Fig. 1, variability within groups is presented in Table 1). The pre-oviposition period differed at the two rearing temperatures from October to January. At the higher temperature (25°C), activation by the tachytelic diapause development was faster (pre-oviposition period was shorter) than at the lower temperature (20°C). Thus, the shortening of the pre-oviposition period

Table 1. Activation of females of *Oxycarenus lavaterae* under a long day photoperiod (18L : 6D). N = 35 for each group. Asterisks indicate pairwise differences in the same month between the two temperatures based on t-tests, with Bonferroni-adjusted levels: * = 0.0056, ** = 0.0011, *** = 0.00011, NS = non-significant.

	Month of transfer from outdoors to laboratory conditions								
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
				20°C					
% of females that started to oviposit	86	86	86	91	86	89	86	86	89
Pre-oviposition period (days)									
mean ± SD	98±49	93±43	77±37	64±30	45±17	26±8	22±9	17±10	10±7
min–max	33–241	29–195	28–146	33–133	21–74	12–41	10–40	7–45	4–31
difference	a	a	b	b	c	d	de	de	e
				25°C					
% of females that started to oviposit	86	86	86	86	86	86	86	86	86
Pre-oviposition period (days)									
mean ± SD	62±26	53±19	42±14	35±11	33±16	25±10	24±9	15±7	9±5
min–max	28–141	25–96	24–79	18–59	12–71	14–52	12–47	6–35	3–22
difference	a	b	c	cd	d	e	e	f	f
Pre-oviposition period, 20 vs. 25°C	**	***	***	***	NS	NS	NS	NS	NS

Table 2. Activation of *Oxycarenus lavaterae* females kept under a short-day photoperiod (12L : 12D) at 25°C. Longevity is measured in terms of number of days after transfer and Fecundity the lifetime fecundity of the females that oviposit (egg number, mean ± standard deviation); no significant differences. N = 40 for each group. Lower case letters indicate means that differ among months (ANOVA, post hoc comparisons: Longevity – Fisher LSD, other variables – unequal N HSD).

	Month of transfer from outdoors to laboratory conditions						
	Dec	Jan	Feb	Mar	Apr	May	Jun
% of females that oviposit	5	10	12.5	40	47.5	55	25
Longevity (days, mean ± SD)	169±33	133±30	110±25	100±19	69±17	44±8	22±6
	a	b	c	d	e	f	g
Pre-oviposition period (days, mean ± SD)	167±4	150±16	106±10	88±10	60±11	37±11	18±5
	a	a	b	b	c	d	e
Fecundity	71±8	50±30	62±24	47±29	36±23	39±27	35±20
Egg clusters	4.5±0.7	3.3±1.7	4.0±1.4	3.2±1.8	1.8±1.1	1.8±0.9	1.8±0.8
	ab	ab	ab	a	b	b	ab
Eggs per cluster	15.8±2.2	15.4±5.3	15.4±4.2	14.8±5.1	19.4±5.5	22.2±8.1	19.3±7.0

during hibernation was steeper at 20°C (0.46 ± 0.03 days per day and only 0.20 ± 0.01 days per day at 25°C). In February, the mean duration of pre-oviposition period was similar at both temperatures (Table 1). From that time onwards, the decrease in the duration of the pre-oviposition period continued at 25°C and followed the same straight line (Fig. 1), while the rate of decrease in the duration of the pre-oviposition period at 20°C was slower than before March and decreased at a similar rate (0.24 ± 0.05 days per day) and reached the same values as recorded at 25°C.

Termination of diapause under a short photoperiod

Most females transferred from outdoors to SD conditions in a laboratory from December to February remained in diapause and did not oviposit despite their long longevity (Table 2). The percentage of females that oviposit increased substantially in March and continued to increase until May, when it was over 50%. There was an eventual decrease in June, connected with noticeable decrease in longevity.

Longevity of females under SD decreased substantially and almost linearly with the time of transfer. Mean decrease was 0.79 ± 0.04 days per day in outdoor conditions. The rate of decrease in the pre-oviposition time was 0.86 ± 0.04 days per day when kept outdoors. Duration of pre-oviposition period was similar to the mean longevity, which means that those females that started to oviposit did so about the time the others were dying at the highest rate. The longevity of females that oviposit was slightly longer than that of those that did not oviposit in each group, especially in March (Table 2).

Mean lifetime fecundity tended to decrease slightly with time of storage, but the difference was statistically insignificant due to high individual variability (Table 2). Females activated from December to March oviposit three to four times before they die, while those activated from April to June oviposit once or twice (Table 2; $F_{6,71} = 5.67$, P = 0.00007). The mean number of eggs per batch was 20 (Table 2) and the maximum 31 (female transferred to the laboratory in April).

Table 3. Size of the clusters of overwintering *Oxycarenus lavaterae* on three trees (horizontal × vertical dimension) in the Czech Republic (48°58´44˝N, 14°28´00˝E) in 2015.

	Size of the clusters on particular dates (cm)						
	Feb 22	Mar 18	Apr 06	Apr 26	May 14	Jun 04	Jun 11
Tree 1	14 × 21	11 × 18	12 × 16	10 × 14	7 × 9	4 × 5	0
Tree 2	10 × 8	10 × 8	11 × 7	10 × 7	9 × 4	0	0
Tree 3	6 × 20	6 × 21	3 × 14	3 × 12	3 × 9	1 × 7	0

Outdoor observations

Large overwintering clusters of adult *O. lavaterae* were observed in South Bohemia on trunks of linden trees in February 2011. These bugs dispersed in May and in October 2011, new overwintering clusters were observed. During the harsh winter of February 2012, these bugs most likely survived by basking in sun during daytime on the south and southwest sides of the trunks. After several cloudy days with severe frosts at night, virtually all bugs died. No bugs were observed during winter 2012/2013. However, in October 2013, there were large aggregations on the same trees. During the winter, they were on the south and southwest sides of the trunks and in March 2014, when temperatures increased to over 20°C, the bugs were on the eastern and in May (maximum temperatures over 30°C) on the northeast side of the trunks. Some of these bugs were observed in the crowns of the trees in May and others were still in the "overwintering" clusters in early June. The trees had fully developed leaves in May and flowered in early June. In spring 2015, the size of the clusters of bugs observed on these trees gradually decreased, due to bugs moving to less sun-exposed places and into the crowns of the trees for feeding (Table 3). A small percentage of the bugs remained in diapause until early June when the lime trees flowered.

DISCUSSION

Diapause strategy

This study revealed that *O. lavaterae* is sensitive to photoperiod throughout hibernation and in late spring. This strategy is reported in only a few species of Heteroptera, such as, *Leptocorisa chinensis* Dallas, 1852 (Heteroptera: Alydidae) (Tachibana & Watanabe, 2007). In this bug, the feeding of overwintered adults is a pre-requisite for egg maturation. The host plant (*Setaria viridis*; Poaceae) is available in mid-June. If they were not sensitive to photoperiod during the winter solstice and activity resumed in early spring the bugs would terminate dormancy long before their host plant became available.

The loss of photoperiodic response is considered a criterion of diapause completion in most Heteroptera, e.g. the seed eaters *Pyrrhocoris apterus* (Hodek, 1971a, 1988) and *Lygaeus equestris* (Solbreck & Sillen-Tullberg, 1981). *Pyrrhocoris apterus* depends on finding the previous years' dry seeds of linden trees (*Tilia* spp.) in early spring. It seems that *O. lavaterae*, which also inhabits linden trees, does not rely on this food source, but waits for linden to flower in late spring. To synchronise with the blossoms, it remains sensitive to day length until late spring. Thus, there is a lack of the commonly used criterion of the end of diapause. It is tempting to conclude that diapause ends in March, when the slopes of the durations of the pre-oviposition periods recorded at two temperatures match (Fig. 1). At that time *O. lavaterae* disperses from overwintering aggregations in Bulgaria. However, the pre-oviposition period would still be rather long (about 25 d) for post-diapause individuals. Field observations in the Czech Republic also indicate that these bugs can still be in diapause in May and June. Thus, it is likely that the diapause strategy of *O. lavaterae* is similar to the rare type described above for the alydid bug *L. chinensis*.

Decrease in the pre-oviposition period an indication of diapause development in *O. lavaterae*

Like most heteropterans inhabiting the temperate regions, adult *O. lavaterae* diapause overwinter. During late autumn, winter and spring, there is a gradual decrease in the duration of the pre-oviposition period measured after transfer to conditions suitable for development.

At 25°C and LD conditions, the decrease in the pre-oviposition period continued almost linearly from October until June, while at 20°C and LD conditions it underwent a change in the rate of decrease in March (Fig. 1). Under SD conditions, a large percentage of the females did not resume development in spring. The continuing decrease in the duration of pre-oviposition period in spring may either indicate further progress with diapause development or a shortening of post-diapause development. Because some of the overwintering bugs remained in clusters (presumably in diapause) till early June, while the others dispersed, there could be a mixture of these two processes.

The decrease in the pre-oviposition period during overwintering (from October to March) was much steeper at 20°C, leading in early March to values equal to those achieved at 25°C. At that time, the higher of the two laboratory temperatures did not produce faster activation (in combination with LD conditions) from dormancy. It is likely that the bug was no longer sensitive to the high temperature in March. Cold storage at 10°C shortened, even more, the pre-oviposition period of *O. lavaterae*.

Photoperiodic response and end of diapause

For the two seed-eating heteropterans, *P. apterus* (Hodek, 1971a, 1988) and *L. equestris* (Solbreck & Sillen-Tullberg, 1981) and many other adult insects that are in diapause when hibernating (Tauber et al., 1986; Danks, 1987; Saulich & Musolin, 2012), the insensitivity to photoperiod is recorded around the winter solstice. This is considered to be an indication of diapause completion/termination (i.e. end of diapause). At that time most females laid eggs after transfer from the field to higher temperatures in a laboratory, both under LD and diapause promoting SD photoperiods. Outdoors they became quiescent due to low temperature or lack of food.

In the case of *L. chinensis* the long-lasting response is seen as an adaptation that keeps this bug inactive until its host plant becomes available. It is unlikely that this is the case for *O. lavaterae* as both its larvae and adults can be reared successfully on the dry seed of *Tilia* trees, thus, like *P. apterus*, it would appear to have food continually available outdoors on the ground under trees. It is more likely that flower buds or young seed are more suitable for egg maturation in females or development of the young instars of *O. lavaterae*.

Alternatively, *O. lavaterae* may retain an adaptation from the area of origin for feeding on herbaceous Malvaceae that are available later in spring (Pericart, 2001) and that linden (*Tilia*) is an alternative host plant in the absence of its preferred host.

Capacity for range expansion

Two congeneric species of the species studied here are pests: *Oxycarenus hyalinipennis* is an important pest of cotton worldwide (Slater & Baranowski, 1994), and *Oxycarenus laetus* is one of the most common and polyphagous pests in Southern Pakistan (Awan & Qureshi, 1996). *Oxycarenus lavaterae* has a similar dietary range (Kalushkov & Nedvěd, 2010) to *O. hyalinipennis* and is therefore a potential pest. Cold hardiness enables winter survival of part of the population in central Europe, but the reproductive potential is large enough to enable the populations to become abundant again in summer (Nedvěd & Kalushkov, in prep.). If the mild winters that occurred in central Europe over the last few years (Brázdil et al., 2023) were to occur in more northern countries, the expansion of the species is likely to continue.

The requirement of a long day photoperiod for the resumption of development recorded in this study seems to be adaptive in the Mediterranean, where this bug must wait for food plants to produce seeds. It did not prevent the spread of this species throughout temperate Europe, where linden seed is available year-round. Moreover, the continual sensitivity to photoperiod in spring is a rare phenomenon deserving further study. Even in northern Europe, these bugs can complete development. If their diapause is not terminated by photoperiod then they may be able to increase the number of generations completed per year even without climate warming (Yakamura & Kiritani, 1998).

This species is native to southern Spain, Near East and north Africa (Pericart, 2001). Investigations of the life history of *O. lavaterae* in its native range are needed. In addition, a search for this bug in northern Europe, helped by citizen science programs (Skuhrovec et al., 2021), should be organized.

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