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Sympatric speciation in *Yponomeuta*: no evidence for host plant fidelity

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Abstract

According to sympatric speciation theory, adaptation to different host plants is expected to pleiotropically lead to assortative mating, an important factor in the reduction of gene flow between the diverging subpopulations. This scenario predicts mating on and oviposition preference for the respective hosts in both the diverging subpopulations and recently originated species. Here, we test both predictions in the oligophagous *Yponomeuta padellus* (L.) and the monophagous *Yponomeuta cagnagellus* (Hübner) (Lepidoptera: Yponomeutidae), two closely related small ermine moth species from the western European clade of *Yponomeuta* for which speciation in sympatry has been proposed. Mating location and adult host acceptance were evaluated under both semi-field (in a large outdoor cage with a choice of host and non-host plants) and field conditions. In the semi-field experiment, only *Y. cagnagellus* showed some preference for mating on its own host (16% of all mating pairs) over non-host plants (3% of all mating pairs). However, in both species, more than 80% of the mating pairs were not formed on a plant but instead on the cage itself. Further examination of the mating site of *Y. cagnagellus* in the field revealed no preference for host plants over non-host plants in the two consecutive years of observation. *Yponomeuta padellus* females, collected from and reared on *Prunus spinosa* L. (Rosaceae), showed an oviposition preference for the alternative host *Crataegus monogyna* Jacq. (Rosaceae) in the semi-field experiment. We thus found no evidence that host-plant fidelity (in terms of mating site) has been the driving force in the speciation process of these *Yponomeuta* species, nor did we find evidence of host race formation in the tested population of the oligophagous *Y. padellus*.

Introduction

Radiations, in which each species in a group of closely related, sympatrically occurring species is specialized on a different host plant, are quite common among phytophagous insects. Examples are aphids of the *Aphis fabae* complex (Raymond et al., 2001), *Ophraella* beetles (Futuyma et al., 1993), tephritid fruit flies of the genus *Rhagoletis* (Feder & Bush, 1989), and *Neochlamisus* leaf beetles (Funk et al., 2002). Such a radiation pattern is indicative of speciation through the formation of host races (i.e., sympatrically living biotypes that differ genetically from one another due to adaptation to different

hosts) (see, for example, Bush, 1975, 1994; Berlocher & Feder, 2002; Drès & Mallet, 2002). Speciation under sympatric conditions requires disruptive selection on a polymorphic trait, such as habitat or host choice, causing groups of individuals within one population to diverge (Coyne & Orr, 2004, and references therein). In addition to disruptive selection, assortative mating is essential for sympatric speciation, as it prevents the continuous formation of intermediate phenotypes between the diverging subpopulations and thus counteracts the homogenizing effects of gene flow (Felsenstein, 1981; Via, 2001). Various theoretical models (Felsenstein, 1981; Dieckman & Doebeli, 1999; Doebeli & Dieckmann, 2000; Doebeli, 2005) have demonstrated that assortative mating plays a crucial role in speciation.

Assortative mating occurs when random mating within a population is restricted as a result of behavioural, temporal,

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or habitat (ecological) isolation (Funk et al., 2002). In the last case, assortative mating is a pleiotropic effect of host fidelity. For example, many phytophagous beetles, such as *Galerucella nymphaeae* L. (Pappers, 2001) and *Neochlamisus* leaf beetles (Funk et al., 2002), have a strong affinity for their host plant, upon which they spend their entire lives. They mate and oviposit on the host, and mating between individuals that share a preference for that host will prevail. This habitat-based assortative mating can thus lead to pre-zygotic reproductive isolation between populations of individuals with different host plant preferences. It has been proposed to be the principal type of assortative mating in many phytophagous insects (Berlocher & Feder, 2002), but the number of studies supporting this view is limited (Funk et al., 2002). Moreover, quite a number of phytophagous insects (e.g., Lepidoptera) do not spend their adult life on the host, thus calling the generality of this speciation mechanism into question. In this article, we use two closely related *Yponomeuta* species (Lepidoptera: Yponomeutidae) to test for mating on the host plant, the predicted signature of a sympatric speciation event following a traditional habitat-based isolation scenario.

The radiation of the nine western European species of the genus *Yponomeuta* has been proposed as an example of sympatric speciation in which host plant shifts have played a key role (Herrebout et al., 1976; Menken, 1981, 1996; Hendrikse & Vos-Buennemeyer, 1987; Menken et al., 1992). The species are largely sympatric, are found on different host plants, and are monophagous with the exception of *Yponomeuta padellus* (L.) (Lepidoptera: Yponomeutidae), which is oligophagous. *Yponomeuta* species, therefore, provide a good model for the study of the host plant fidelity that is required for habitat-based assortative mating and its role in the (sympatric) speciation of these moths. Two of the species, *Yponomeuta cagnagellus* (Hübner) (Lepidoptera: Yponomeutidae) and *Y. padellus*, are quite closely related (H Turner, N Lieshout, W van Ginkel & SBJ Menken, unpubl.), hybridize easily, and produce viable offspring in the laboratory (Menken, 1980; Roessingh et al., 1999; Hora et al., 2005); in nature they rarely do so (Arduino & Bullini, 1985). The two species have non-overlapping host plant preferences: *Y. cagnagellus* is strictly monophagous on *Euonymus europaeus* L. (Celastraceae), while *Y. padellus* is oligophagous on *Prunus spinosa* L., *Crataegus monogyna* Jacq., *Sorbus aucuparia*, and *Amalanchier lamarckii* F. G. Schroed. (all Rosaceae). If host plant fidelity has played an important role in the evolution of reproductive isolation of *Yponomeuta* species, then we would expect to find that host plant fidelity is still apparent in the closely related *Y. cagnagellus* and *Y. padellus*. To test this prediction, mating site selection was evaluated under semi-field conditions, as well as in the field.

Experiments of mating site preference were designed to detect a predicted signature of a past sympatric speciation event. It has, however, also been suggested that one of these two species, the oligophagous *Y. padellus*, is in an early stage of host race formation. If this is the case, genetically distinct subpopulations are expected to be present on the different hosts. The population structure of *Y. padellus* has been extensively studied in this respect. Menken (1981, 1989) found restricted levels of gene flow in particular populations of *Y. padellus* in The Netherlands. However, Raijmann (1996) and Raijmann & Menken (2000) in later studies conducted partly on the same populations as those of Menken (1981), found only weak indications of restricted gene flow, and Drès & Mallet (2002), thus, rightly concluded that there is not yet enough evidence for host race formation in *Y. padellus*.

One of the early signatures of host race formation would be a preference to oviposit on a particular host in the range of acceptable plants for oviposition. Several experiments on the mating and oviposition behaviour of *Y. padellus* have been conducted (Hendrikse, 1990; Kooi et al., 1991; Geerts et al., 2000). Some of these indicated host plant preference, while others did not. However, all of these experiments were carried out in small arenas under laboratory conditions. Funk et al. (2002) found that the leaf beetle *Neochlamisus bebbianae* (Brown) exhibited much stronger host plant fidelity when tested in larger arenas that provided a better imitation of natural conditions. Furthermore, Turanli & Schaffner (2004) found a more pronounced host plant fidelity with respect to oviposition in *Tinthia myrmosaeformis* (Lepidoptera: Sesiidae) in an open field set-up than in laboratory and cage experiments. Therefore, more natural experiments are needed to establish the true degree of host plant fidelity in *Yponomeuta* moths. Here we evaluate, in addition to the mating location, the host acceptance behaviour of *Y. cagnagellus* and *Y. padellus* in choice experiments with host and non-host plant in a large outdoor cage.

Materials and methods

Rearing

Yponomeuta cagnagellus and *Y. padellus* were collected as fourth or fifth instars from *E. europaeus* (in Amsterdam; 52°21′N, 4°57′E) and from *P. spinosa* (in Leiden; 52°8′N, 4°29′E), respectively (both in The Netherlands). Caterpillars were kept in a climate room at 60% r.h., 19–22 °C, and L17:D7, and were fed ad libitum freshly picked leaves of their respective host plants (from 1–2-year-old plants grown in the greenhouse). All larvae were reared in plastic 10-cm Petri dishes until pupation; a piece of moistened cotton wool kept their food source fresh. Pupae

were transferred to glass tubes (8 cm in length × 2.1 cm in diameter) and maintained under the same conditions as the larvae. After eclosion, moths were kept in the glass tubes until they reached sexual maturity, at which point they were used in the experiments.

Semi-field experiment: mating substrate and oviposition preference

Two cages (one for each species) were set up in a small open field on the campus of the University of Amsterdam. Each cage measured 4 × 5 × 2 m, and consisted of a steel frame with walls and ceiling made of a white nylon net (mesh size 1 mm). The cage for *Y. cagnagellus* had black porous mulching plastic on the ground, while the floor of the cage for *Y. padellus* was covered with short grass. In each cage, 12 plants in plastic pots were arranged in a circle with a diameter of about 3 m. The plants were 2 years old, of equal height (approximately 70 cm), and placed about 70 cm from one another. We alternated a *P. spinosa* plant with an *E. europaeus* plant and then a *C. monogyna* plant, and this pattern was repeated four times to complete the circle of 12 plants. There were thus six *Y. padellus* host plants (three *P. spinosa* and three *C. monogyna* plants) and six *Y. cagnagellus* host plants (six *E. europaeus* plants). This set-up guaranteed that *Y. padellus* and *Y. cagnagellus* host and non-host plants always alternated. The experiment was conducted from 24 to 27 June 2003, the period during which the first adults of both *Yponomeuta* species could be found in the field. On the first evening of the experiment, 220 approximately 1-week-old individuals (110 males and 110 females) of *Y. padellus* were released in the middle of cage 1. This was repeated on the third evening, thus, bringing the total to 440 *Y. padellus* moths. As for *Y. cagnagellus*, 175 female and 175 male moths of approximately 2 weeks of age were released into the middle of cage 2 on the first evening followed by another 125 females and 125 males on the third evening, resulting in a total of 600 *Y. cagnagellus* moths in this cage. Although this is a considerable density, it is not uncommon to find such large numbers of adults in the field. Both species are highly gregarious, pupate on or under their host, and show only limited dispersal. Moths were introduced into the cages between 20:00 and 23:00 hours. During the 4 nights of the experiment, the cages were checked for mating pairs approximately once an hour between 20:00 and 09:00 hours. In this way, mating pairs would not be missed, because in both species, mating with a virgin male lasts between 3 and 6 h. A red flashlight was used to observe the moths without disturbing them during the scotophase. The positions of all mating pairs were recorded, after which the pairs were collected and kept in glass vials at room temperature near a window (thus, experiencing natural light conditions). Once they had separated, they were returned to the outdoor cage the following day in order to allow the females to lay eggs. After the summer, when all moths in the cages had died, all plants from both cages were checked twice for egg batches.

Field observations on *Yponomeuta cagnagellus*

The field plot was a dune area of approximately 120 m² in the 'Kennemerduinen' in The Netherlands (52°23′N, 4°35′E). Field observations on mating pairs of *Y. cagnagellus* and their mating site were made from 23 July to 15 August 2003 between 08:30 and 09:35 hours, on a total of 7 mornings. In 2004, field observations were made from 19 July to 6 August between 08:20 and 09:30 hours on a total of 10 mornings. Moths were observed to pair up around dawn and these pairs remained together for several hours, similar to what we had observed in the laboratory. In both years, we checked two *Quercus robur* L. (Fagaceae), one *Ulmus carpinifolia* Gled. (Ulmaceae), four *E. europaeus*, and one *C. monogyna* plant every 2 or 3 days. These plants form a representative sample of the bush and tree species in the area where *Y. cagnagellus* occurs. Bushes and trees grew on both sides of a sandy path, at distances of 1–5 m from each other. The sampled bushes were selected for their equal volume (i.e., 4.5 m³). Most plants were approximately 2 m high, not counting the trunk, and 1.5 m wide and deep. On the much larger *Quercus* trees, an isolated overhanging branch of about the same volume was checked. We placed a dark-coloured cloth under a tree and shook it forcefully several times until all moths that were in copula had fallen out of the plants. Moth pairs were easily visible due to their white wings. We checked the plants carefully after shaking them, but we never found any couples that had managed to hold on. The order in which the bushes were checked alternated from day to day to avoid any bias.

Results

Host-plant fidelity and time of mating in the semi-field experiment

In total, 164 *Y. padellus* mating pairs (220 virgin females were available) and 70 *Y. cagnagellus* mating pairs (300 virgin females were available) were formed in the outdoor cages during the 4 nights of observation. For both species, it appeared that most pairs (81% for *Y. cagnagellus* and 87% for *Y. padellus*) were situated on the side walls and ceilings of the cages, although in *Y. padellus* a few were found on the bottom (Figure 1). The moths were evenly spread over the walls and the ceiling, except that few moths were found on the net within 0.5 m of the ground. Only a small percentage of mating pairs, namely, 16% for *Y. cagnagellus* and 4% for *Y. padellus*, were found on their respective host plants. Only in *Y. cagnagellus* was this percentage significantly higher on host plants than on
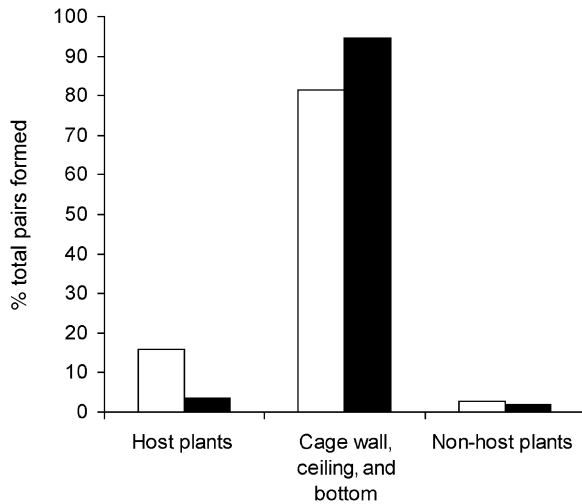
Figure 1 Location of mating pairs of *Yponomeuta padellus* (black bars) and *Yponomeuta cagnagellus* (white bars) formed during 4 nights in the semi-field experiment.

non-host plants (binomial test: P = 0.01). One of the mating pairs of *Y. padellus* was encountered on *C. monogyna*, five pairs were on *P. spinosa*, and three were on the non-host *E. europaeus*. Although the number of pairs on the plants were too low for a meaningful statistical analysis, it was obvious that there is no clear preference for any plant species. No difference in the distribution pattern of mating pairs between nights was observed for either species (data not shown). Direct observations made during the night and early morning showed that female moths of both species called from a fixed location, leading males to approach the female; this was followed by courtship behaviour and mating on the spot. Whether it was disturbed or not, a pair in copula was never seen to move more than a few centimetres after mating had been initiated. A pair in copula cannot fly; the male and the female usually keep a firm grip on the substrate, so that only a vigorous shaking of the substrate will cause a pair to fall to the ground. Both *Y. cagnagellus* and *Y. padellus* were sexually active and formed mating pairs mostly at sunrise

(ca. 04:30 hours). However, a small number of both species mated at around sunset or at the beginning of the night. Four *Y. cagnagellus* pairs began to mate between 20:40 and 22:30 hours. One, four, five, and three *Y. padellus* pairs began to mate between 22:25–00:15, 23:10–00:00, 01:00–02:00, and 02:00–02:20 hours, respectively, during the 4 nights of observation.

Oviposition preference in the semi-field experiment

Yponomeuta padellus females did not evenly distribute egg batches across the two host plants, *C. monogyna* and *P. spinosa*. We found an oviposition preference for *C. monogyna* (23 egg batches on *C. monogyna* vs. eight on *P. spinosa*; binomial test: P = 0.01; Figure 2), even though all of the *Y. padellus* moths used in the experiment eclosed on *P. spinosa* plants and ate only this plant species throughout their larval lives. Surprisingly, three egg batches were oviposited on the non-host *E. europaeus*; *Y. padellus* larvae cannot survive on this host and die before they moult to the second instar (R Kooi, pers. comm.). *Yponomeuta cagnagellus* females oviposited 79 egg batches on their host *E. europaeus* and one egg batch on the non-host *C. monogyna*.

Field observations on *Yponomeuta cagnagellus* mating pairs

In both years of observation, more mating pairs in the field were found on non-host plants than on host plants (Table 1). It is unlikely that this can be explained by the availability of substrate, as both plant groups had about an equal volume and thus probably a similar surface in the field area. After combining the data of both years of observation, we found no preference for *Y. cagnagellus* moths to mate on their host plant *E. europaeus* (one-sided binomial test: P = 0.99). In fact, a significantly lower number of mating pairs was found on the host plant than on the non-host plants (12 vs. 36; binomial test: P = 0.0007).

Discussion

In this study, we searched for signatures of sympatric speciation by investigating host plant fidelity, that is,

Table 1 Numbers of *Yponomeuta cagnagellus* mating pairs encountered on host plants (*Euonymus europaeus*) and non-host plants (*Crataegus monogyna*, *Quercus robur*, and *Ulmus carpinifolia*) in the field plot in 2003 and 2004. The number of plants sampled are given in parentheses

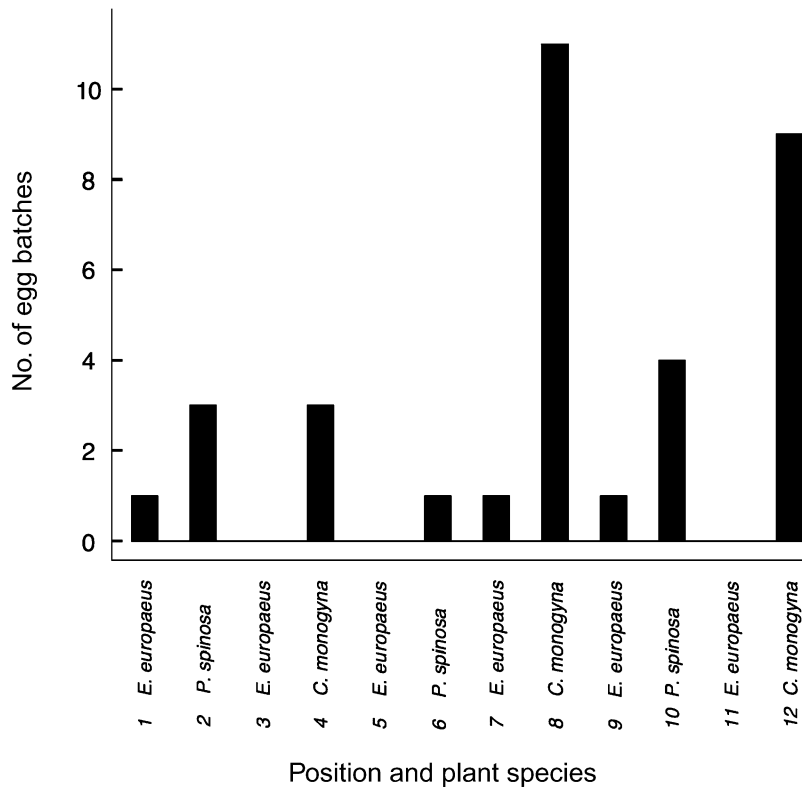| Year	*C. monogyna* (1)	*E. europaeus* (4)	*Q. robur* (2)	*U. carpinifolia* (1)	Total number of pairs on host plants	Total number of pairs on non-host plants
2003	1	3	12	1	3	14
2004	1	9	11	10	9	22
Total	2	12	23	11	12	36

Figure 2 Position of 34 egg batches of *Yponomeuta padellus* on *Crataegus monogyna*, *Prunus spinosa*, and *Euonymus europaeus* in the semi-field experiment.

mating site and oviposition site selection. Specifically, after a recent sympatric speciation event that was facilitated by habitat-based assortative mating, mating on the host is predicted. Before such an event, host populations in oligophagous species are expected to show differential oviposition preferences, that is, if *Y. padellus* consists of host races, we expected to find a mating and oviposition preference for *P. spinosa*, the host plant from which we collected *Y. padellus* and on which it was reared.

Habitat-based assortative mating

Neither of the species showed strong host fidelity for mating site. In the semi-field experiment, the majority of *Y. cagnagellus* mating pairs were found on the cage itself and not on the plants. We did find a preference for mating on the host in the 19.4% of *Y. cagnagellus* pairs that were found on plants: 81% mated on the host. It cannot be fully excluded that these low levels of host fidelity during oviposition are an artefact caused by the cage. However, mating pairs in the field did not show a preference either, as only 25% of the pairs were found on host plants. In the semi-field experiment, *Y. padellus* mated also mainly on the cage itself. The comparison of (the small) numbers of mating pairs between host and non-host plants did not indicate a preference. Unfortunately, we have no field data

for *Y. padellus*. Nonetheless, host plant fidelity in the field seems unlikely, given the fact that even in the closely related *Y. cagnagellus*, for which we found significantly more pairs on host than on non-host plants in the semi-field experiment, no such preference was observed in the field. This study thus fails to provide support for mating on the host, the predicted signature of sympatric speciation driven by habitat-based assortative mating. Although sample size is small and more data are clearly needed to confirm the results, host fidelity is such a key feature of this type of speciation mechanism that a strong signal would have been expected. Therefore, the result of this study questions the importance of host fidelity in the radiation of the European clade of *Yponomeuta* moths as was suggested by, for instance, Menken & Roessingh (1998).

Oviposition preference

We assessed oviposition site preference in the oligophagous *Y. padellus* in order to see if host races exist in this species. In the semi-field experiment, *Y. padellus*, which had been collected from and continuously reared on *P. spinosa*, was given the choice between *P. spinosa* and its other common host, *C. monogyna*.

Our study provided no evidence for host race formation in *Y. padellus*. In fact, *Y. padellus* moths from *P. spinosa*

unexpectedly laid significantly more egg batches on *C. monogyna*. Due to their small size (<1 mm), young *Yponomeuta* larvae cannot easily switch host plants. Therefore, as long as the mother exhibits no oviposition preference, the larvae will grow randomly on different host plants, and thus the genes for preference for and adaptation to a particular host plant will not become linked (Thompson & Pellmyr, 1991); this will prevent the successful formation of host races. Kooi et al. (1991) demonstrated that female *Y. padellus* showed no preference for ovipositing on their own host plant in experiments conducted in a small cage. We performed this experiment under more natural conditions (in a field cage 100× the size of the laboratory cages); however, this did not change the outcome.

Host odours

Host plant odour is not required to achieve successful mating in the laboratory (Hendrikse, 1990). However, several laboratory studies have indicated that *Yponomeuta* moths respond to the odours of the host plant on which they have fed as larvae. For example, Hendrikse & Vos-Buennemeyer (1987) found that *Y. cagnagellus* males remained for a significantly longer period in the part of a wind tunnel where the host plant odour was present. In a similar experiment, however, no such effect was found for *Y. padellus* (Hendrikse & Vos-Buennemeyer, 1987). These authors also found a stimulating effect of host plant on calling behaviour (i.e., pheromone emission to attract males) of *Y. padellus* females. Furthermore, they showed that mature *Y. padellus* females preferred an area with host plant odour for calling. However, even though we encountered some host plant fidelity in *Y. cagnagellus* under the more natural conditions of the semi-field experiment, this did not lead to a clear preference for calling and subsequent mating on the host plant. Apparently, other factors play an important role for females in their choice of a calling site. Females of both *Yponomeuta* species might select for a location that is suitable for pheromone dispersion, and thus for a particular height in the vegetation associated with a particular humidity and wind speed. It is also possible that protection from the weather or predators influences their choice of mating location.

Habitat isolation vs. behavioural and temporal isolation

In many Lepidoptera, females mate with males on the day that they emerge from their pupae (Scott, 1972; Ramaswamy et al., 1997). If the pupae are on the host, this would then facilitate habitat-based assortative mating. In *Yponomeuta* moths, however, this is not the case. Caterpillars do pupate on or under the host plant on which they have fed but moths are not yet sexually mature upon eclosion (Hendrikse, 1979). After eclosion, *Yponomeuta*

moths disperse from their host plant to collect nutrients (we have observed many *Yponomeuta* moths feeding on leaf exudates of *U. carpinifolia* at the field site), or to avoid predation and parasitism, which may be higher at their pupation sites. We cannot exclude the possibility that assortative mating on the host plant (habitat isolation) has played a role in the past at the start when divergence processes had only just gotten underway. It is possible that habitat-based assortative mating was lost once another reproductive isolation mechanism (e.g., pheromones, see below) had evolved. Nonetheless, this implies that habitat isolation was possibly not the foremost mechanism that drove speciation of *Y. cagnagellus* and *Y. padellus.*

Although the initial focus of sympatric speciation processes in phytophagous insects was on habitat isolation (Bush, 1975, 1994; Rice, 1987), temporal isolation and changes in pheromone communication and courtship behaviour have been found to be important in establishing assortative mating. In the best documented example of host race formation, the apple maggot fly, *Rhagoletis pomonella* (Walsh), temporal (i.e., allochronic) isolation played a key role (Feder & Filchak, 1999). Females eclose and mature synchronously with the ripening of the fruits of apple and hawthorn, the hosts of the two host races, and this results in a 10-day difference in the mating season of the host races. The case of the European corn borer, *Ostrinia nubilalis* (Hübner), shows that temporal isolation combined with behavioural isolation can provide strong reproductive isolation (Thomas et al., 2003): differences in pheromone composition (behavioural isolation) and partially overlapping mating seasons (temporal isolation) have lead to genetically distinct host races on maize and mugwort (Bethenod et al., 2005). Some differences in courtship behaviour, such as differences in wing fanning patterns between males, were found between several species of *Yponomeuta* (Hendrikse, 1990). Furthermore, absence of interspecific attraction of four out of eight *Yponomeuta* species was found in a wind tunnel experiment (Hendrikse, 1986). Löfstedt et al. (1991) subsequently showed that all nine western European *Yponomeuta* species have species-specific sex pheromone blends, with the exception of two species, namely, *Yponomeuta evonymellus* (L.) and *Yponomeuta sedellus* Treitschke, which are completely isolated temporally. These different pheromone systems apparently play and probably have played an essential role in reproductive isolation between *Yponomeuta* species.

Temporal isolation has not received much attention in studies on *Yponomeuta*. The results of laboratory experiments by Hendrikse (1979), as well as those of our semi-field experiment, show a large overlap between *Y. padellus* and *Y. cagnagellus* in circadian mating behaviour. However, similar to earlier suggestions by Hendrikse (1979), we have

strong indications that the mating season of *Y. cagnagellus* starts later in the year, when only few sexually active *Y. padellus* moths are left (Bakker et al., 2008). In the field, eclosion takes place around the same date for *Y. cagnagellus* and *Y. padellus* (Herrebout et al., 1975). However, laboratory experiments showed that *Y. cagnagellus* becomes sexually mature up to 10 days later and lives much longer than *Y. padellus* does (Hendrikse, 1979; Hendrikse & Vos-Buennemeyer, 1987); this can lead to partially non-overlapping mating seasons. Therefore, it is plausible that temporal isolation played a role in reproductive isolation between these two small ermine moth species.

Acknowledgements

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