

Heterospecific courtship, minority effects and niche separation between cryptic butterfly species

M. FRIBERG*†, O. LEIMAR* & C. WIKLUND*

*Department of Zoology, Stockholm University, Stockholm, Sweden

†Department of Plant Ecology and Evolution, Evolutionary Biology Centre (EBC), Uppsala, Sweden

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Abstract

Species interacting in varied ecological conditions often evolve in different directions in different local populations. The butterflies of the cryptic *Leptidea* complex are sympatrically distributed in different combinations across their Eurasian range. Interestingly, the same species is a habitat generalist in some regions and a habitat specialist in others, where a sibling species has the habitat generalist role. Previous studies suggest that this geographically variable niche divergence is generated by local processes in different contact zones. By varying the absolute and relative densities of *Leptidea sinapis* and *Leptidea juvernica* in large outdoor cages, we show that female mating success is unaffected by conspecific density, but strongly negatively affected by the density of the other species. Whereas 80% of the females mated when a conspecific couple was alone in a cage, less than 10% mated when the single couple shared the cage with five pairs of the other species. The heterospecific courtships can thus affect the population fitness, and for the species in the local minority, the suitability of a habitat is likely to depend on the presence or absence of the locally interacting species. If the local relative abundance of the different species depends on the colonization order, priority effects might determine the ecological roles of interacting species in this system.

Introduction

The direction of evolution in the same interacting species is often repeated in different local populations with similar ecology (Schluter *et al.*, 2004; Colosimo *et al.*, 2005; Wood *et al.*, 2005), whereas the evolutionary process can be more unpredictable if the local ecology differs between different areas (Thompson & Cunningham, 2002; Thompson, 2005). In this study, we investigate a classical question in ecology and evolution, that of the *raison d'être* of niche separation between closely related species living in sympatry, and explore whether minority effects caused by heterospecific sexual interference is a potential driver for the niche separation of closely related species.

In systems where the common resource is ample, the impact of interspecific resource competition on niche

separation is likely reduced, and niche divergence and competitive exclusion is instead driven by genetic drift and non-resource related selection (reviewed in Gordon, 2000). This might especially apply to phytophagous insects that often experience much lower population densities than the density of their host plant resource (Lawton & Strong, 1981), which means that they are restricted for reasons other than resource competition, such as predation (Roughgarden & Feldman, 1975) or parasite pressure (Yan, 1996). The actual process of niche partitioning is, however, notoriously difficult to study as the interacting species are predicted to reach an equilibrium state soon after the onset of competition, leaving only 'the ghost of competition past' (Connell, 1980), with species being sorted into different ecological niches.

At a population level, important examples of non-resource-related fitness costs of sharing an ecological niche with a closely related species could be the negative impact of premating investment in heterospecific courtship and mating harassment, as well as post-mating costs of hybrid inviability or sterility (Ord *et al.*, 2011).

Correspondence: Magne Friberg, Department of Plant Ecology and Evolution, Evolutionary Biology Centre, Norbyvägen 18D, S-752 36 Uppsala, Sweden.

Tel.: +46 18 471 2855; fax: +46 8 16 77 15;

e-mails: magne.friberg@zoologi.su.se; magne.friberg@ebc.uu.se

The mating preferences of closely related sympatric species are thus predicted to undergo reinforcement, which eventually results in a pattern of reproductive character displacement and assortative mating (Butlin, 1987; Howard, 1993; Liou & Price, 1994; Arnold *et al.*, 1996; Servedio & Noor, 2003). Alternatively (or in parallel), selection from sexual interactions can result in ecological character displacement (Slatkin, 1980; Doebeli, 1996) if individuals preferring a habitat free from the other species are favoured by selection. Recently, increased attention has been paid to measuring the fitness effects of heterospecific courtship interactions (reviewed in Gröning & Hochkirch, 2008). A meta-analysis showed that the level of species recognition is context dependent and the ability to distinguish con- and heterospecifics can often be asymmetrically distributed between the interacting species and sexes (Ord *et al.*, 2011). Studies that assess the fitness costs of heterospecific courtship are rare (Nielsen & Watt, 2000; Gröning *et al.*, 2007; Hochkirch *et al.*, 2007) and are often focused on the costs or benefits of hybridization rather than the cost of the pre-mating sexual interference (e.g. Pfennig, 2007; Wiley *et al.*, 2007; Hochkirch & Lemke, 2011).

Here, we study two sympatric butterfly species that show evidence of strong reproductive isolation, but incomplete and asymmetric sexual isolation, with males spending a substantial part of their time performing costly and elaborate courtships for heterospecific females, and females exclusively accepting conspecific males as mates (Friberg *et al.*, 2008a; Berger *et al.*, 2012a; V. Dincă, C. Wiklund, V. A. Lukhtanov, U. Kodandaramaiah, K. Norén, L. Dapporto, N. Wahlberg, R. Vila & M. Friberg, unpublished data). Our aim is to assess the female fitness costs of a lack of male selectivity and the potential for these costs to result in divergent selection for habitat niche separation, rather than favouring the evolution of species recognition also among males.

After being considered a single species, the wood white butterfly *Leptidea sinapis* has been divided twice; first in 1988 into also including the sibling species *Leptidea reali* (Réal, 1988; Reissinger, 1989), and again in 2011 on the discovery of an additional cryptic species, *Leptidea juvernica* (Dincă *et al.*, 2011). So far, no heterospecific mating has been observed despite the monitoring of numerous heterospecific courtship occasions both in laboratory settings (Freese & Fiedler, 2002; Friberg *et al.*, 2008a; Dincă *et al.*, unpublished data) and in the field (Friberg *et al.*, 2008a; Berger *et al.*, 2012a). A growing body of genetic data also shows that hybridization is at most very rare, and the species boundaries are well supported by both nuclear and mitochondrial data (Dincă *et al.*, 2011). *Leptidea reali* is restricted to Spain, Southern France and Italy, whereas *L. juvernica* is distributed across the rest of Europe from Ireland in the west, the Nordic countries and Russia in the north to Caucasia in the east. *Leptidea*

sinapis is present across the entire *L. reali* and *L. juvernica* distribution (Dincă *et al.*, 2011; Dincă *et al.*, unpublished data), and often collected at the same sites as its sibling species (reviewed in Nelson *et al.*, 2011), whereas the evidence so far suggests that the distributions of *L. reali* and *L. juvernica* do not overlap (Dincă *et al.*, 2011; Nelson *et al.*, 2011).

Leptidea sinapis and its sympatric sibling species have only partly partitioned their niches. Interestingly, this niche separation has been reported to have taken different directions in different parts of Europe, as the interacting species swap habitat specialist and generalist roles between different regions. For instance, in some areas, *L. juvernica* is a meadow specialist and *L. sinapis* a forest–meadow generalist, and in other areas the roles are reversed (reviewed in Friberg *et al.*, 2008b; Nelson *et al.*, 2011; compiled in Table 1, see Data S1 in the Supporting Information for a comprehensive description). The genetic studies suggest that the mosaic pattern of niche separation is likely generated by local processes, as populations of similar habitat-utilization pattern are not each other's closest relatives at the within-species level (Dincă *et al.*, unpublished data). Previous studies also indicate that the varying habitat partitioning is not a result of differing female host plant preferences, because both *L. sinapis* and *L. juvernica* prefer meadow-associated host plants, at least in subpopulations located in Germany (Freese & Fiedler, 2002) and Sweden (Friberg *et al.*, 2008b,c; Friberg & Wiklund, 2009). Furthermore, niche separation is most likely uninfluenced by interspecific larval food competition as host plants typically are ubiquitous at sites containing *Leptidea* populations (Friberg *et al.*, 2008b).

Instead, the selection pressure that generates niche separation could be the long-lasting and elaborate courtship ritual (Wiklund, 1977; Friberg *et al.*, 2008a; see Fig. S1). In nature, most *Leptidea* females mate only once (M. Friberg & C. Wiklund, unpublished data), whereas a fraction of long-lived individuals can mate twice or even three times across their lifespan, which is estimated to average between 5 and 7 days (recorded maximum = 26 days; Friberg *et al.*, 2008b; Berger *et al.*, 2012a). The typical courtship ritual starts with a male detecting a female in flight. He pursues her until she alights in the vegetation so that he can settle in front of her. Thereafter, he starts oscillating his proboscis sideways (Fig. S1), which continues either until the female lowers her abdomen in an acceptance signal, or until the male terminates courtship and flies away. The courtship usually lasts a few minutes, but sometimes as long as 45 min (Wiklund, 1977; Friberg *et al.*, 2008a). Females do not perform any rejection behaviours, but on rare occasions, females try to escape an unwanted courtship by taking off in an ascending flight. This behaviour only rarely pays-off, because most often the male has no problem to follow the female (M. Friberg & C. Wiklund, unpublished data). The long-lasting male

Table 1 Local abundance (WS, R), niche width (G, S) and habitat-utilization patterns (WL, M, DM, CL, HR, ES, HAM, LAM) of *Leptidea sinapis*, *Leptidea juvernica* and *Leptidea reali* in different parts of Europe based on the literature.

Area	Distribution	Species	Abundance/niche width	Habitat	Reference
Catalonia	Sympatric	<i>L. sinapis</i>	WS/G	WL, M, DM	Vila <i>et al.</i> (2003)
		<i>L. reali</i>	R/S	HAM	
Czech Republic	Sympatric	<i>L. sinapis</i>	R/S	M, CL/ES	Beneš <i>et al.</i> (2003)
		<i>L. juvernica</i>	WS/G	WL, HAM, HR	
France	Sympatric	<i>L. sinapis</i>	WS/G	WL, LAM	Amiet (2004)
		<i>L. juvernica/L. reali</i>	R/S	HAM	
Finland	Allopatric	<i>L. sinapis</i>	WS/G	WL, M, CL	Kullberg <i>et al.</i> (2002)
Great Britain	Allopatric	<i>L. sinapis</i>	R/G	WL, M HR	Jeffcoate (2006)
Ireland	Allopatric	<i>L. sinapis</i>	R/S	M	Nelson <i>et al.</i> (2011)
		<i>L. juvernica</i>	WS/G	WL, M, HR	
Poland	Sympatric	<i>L. sinapis</i>	WS/G	WL, M	Sachanowicz <i>et al.</i> (2011)
		<i>L. juvernica</i>	WS/G	WL, M	
Sweden	Sympatric	<i>L. sinapis</i>	WS/G	WL, M, CL	Friberg <i>et al.</i> (2008b,c)
		<i>L. juvernica</i>	WS/S	M	

WS, widespread; R, rare; G, generalist; S, specialist; WL, woodland; M, meadows; DM, dry meadows; CL, clearings; HR, hedgerows; ES, early succession areas; HAM, high-altitude meadows; LAM, lower altitude meadows.

courtships have been proposed to impose time costs both on males and females (Friberg *et al.*, 2008a; Berger *et al.*, 2012a), but the alternative female strategy, to accept mating to avoid the long-lasting courtship, would be even more costly, as the average mating lasts for over an hour, and if the male is recently mated, the time in copula can exceed 10 h (M. Friberg & C. Wiklund, unpublished data).

Engaging in heterospecific courtships is probably affecting the sexes asymmetrically as both pre-mating opportunity costs and post-mating hybridization costs are predicted to often be larger for females than males (Parker & Partridge, 1998; Barry & Kokko, 2010). Reproductive interference has been suggested to be a driver of habitat niche separation (Hochkirch *et al.*, 2007), and the *Leptidea* male inability to distinguish between con- and heterospecific females could impose a large cost, especially on the species in the local minority. Females of this species will be more likely to be caught up in interactions with males of the more common species, and the locally rare males will spend a substantial proportion of their courtship attempts with heterospecific females. Thus, individuals that utilize a habitat that minimizes the risk of meeting heterospecifics and simultaneously maximizes the chances of meeting conspecifics will be favoured by selection.

In this study, we have experimentally manipulated the absolute and relative population densities of *L. sinapis* and *L. juvernica* in large outdoor cages. Thereby, we test the hypothesis that habitat quality depends on the presence or absence of the other species, and assess the courtship-induced female fitness costs of being in the local minority. The study shows that the chance for individual females to become mated depends on the local community in terms of the relative densities of

the interacting species. These data are in agreement with the hypothesis that heterospecific courtship is a selective agent for niche separation in this system. Accordingly, we present a scenario of how being in the local minority can select for habitat specialization, which can result in the formation of a mosaic pattern of niche separation across the joint distribution of sympatric species.

Materials and methods

We set up an outdoor experiment in two large, semicylindrical, cages (30 m long, 8 m wide and with a radius of 4 m; Fig. S2) that were located at Stockholm University's Research Station at Tovetorp approximately 100 km south of Stockholm (58°58' N, 17°09' E). The cages were positioned in an east–west direction on a meadow, located in a *Leptidea* habitat where heterospecific courtships are common during the joint spring flight period of *L. juvernica* and *L. sinapis* (Friberg *et al.*, 2008a). Each cage was subdivided into two 14-m-long compartments. The grass that covered the cage floor was mowed once a week to make the ground less hospitable for spiders and other predators, and to facilitate observations of butterfly behaviour. In each compartment, we placed 14 nectar plants (*Kalanchoe* sp.) that were sprayed with sugar solution on the morning of each experimental day. The experiments took place between 10.00 and 15.00 on sunny days, that is during the daily period when *Leptidea* butterflies are most active at these latitudes (Friberg *et al.*, 2008c), between 29 June and 23 July in 2008, and 30 May and 15 July in 2009.

The butterflies derived from laboratory populations of *L. juvernica* and *L. sinapis* that were started in 2003, and to which new genetic material has been added every summer in the form of offspring from females collected

at two field sites in south central Sweden (Riala and Kronängen, see e.g. Friberg & Wiklund, 2009). Larvae were reared in species-specific groups of five in 1-L jars with *ad libitum* access to the larval host plant *Lotus corniculatus* under daylight and temperature conditions that resulted in direct development (22 : 2 h day : night, 25 °C). Adults were sexed at eclosion, individually labelled with marker pens on the dorsal side of their right hind wing, and stored in a cold room maintaining 10 °C until transported to the experimental site in field coolers. They were unmated at the start of a trial.

Each trial occupied 1 day and started at 10.00 in the morning with the release of butterflies into each of the four compartments. We describe the species distribution in a trial, from the point of view of a focal species, as $X : Y$, where X is the number of pairs of the focal species and Y the number of pairs of the other species. There were four different treatments. The first and second treatment simulated a situation with a skewed species distribution of five males and five females of one species, and one male and one female of the other species. This was replicated on 13 occasions with *L. sinapis* being in the minority (1 : 5) and *L. juvernica* in the majority (5 : 1), and 12 times with *L. sinapis* being in the majority (5 : 1) and *L. juvernica* in the minority (1 : 5). In a third treatment, we released three males and three females of each species into the experimental compartment (3 : 3), and in the last treatment only a single conspecific couple was released into the compartment (1 : 0; for sample sizes, see Table 2). Treatments were randomly assigned to the different cage compartments and distributed evenly over the different experimental days. After release, cages were continuously searched for butterflies *in copula*, as well as for butterflies missing due to predation (most often from spiders) or escape (escapees were only rarely spotted outside the cage and were then recaptured and released into the cage). When a certain butterfly individual was found dead or was missing in a cage, we released a new individual of that species and sex to keep the sex and species ratios intact. At 15.00, all butterflies were recaptured, and the experimental trials were terminated. All matings were noted, and females were later dissected for spermatophores to assess whether matings

had occurred that had not been observed during the experiment.

Statistical analysis and specific predictions

By applying the four experimental treatments, we can disentangle effects of both absolute conspecific density (conspecifics m^{-2}) and the relative species density (conspecifics/total number of con- and heterospecific butterflies) on the female probability of mating during a trial. If a low conspecific density *per se* lowers the probability of finding a mating partner (this would be an Allee effect; Stephens *et al.*, 1999), we expect females to be least likely to mate in the two conditions when they share the cage with a single conspecific male (1 : 0 and 1 : 5), and that the mating probability increases with conspecific density (3 : 3 and 5 : 1). If the mating probability instead is frequency dependent and mainly determined by the relative species density, we expect a similar pattern with an increasing proportion of matings from the 1 : 5 to the 3 : 3 and 5 : 1 conditions, but with the important exception that mating success should be highest in the 1 : 0 condition when a female shared the cage with a single conspecific male and no heterospecific males or females.

We analysed the effects of con- and heterospecific density on individual female mating success in mixed-model logistic regressions. We used the *lmer* function in the *LME4* package in version 2.14.0 of the statistical program R (R Development Core Team, 2011), and further supplemented the mixed model by Bayesian MCMC model fitting, using the *MCMCGLMM* function in version 2.15 of the *MCMCGLMM* package (Hadfield, 2010) in R. Our approach was to first use *lmer* to select the best-fitting model, measured using the Akaike information criterion (AIC). The *lmer* models had the number of mated females in a trial (of the total females of that species in the trial) as a binomially distributed response variable and a logit link function. As independent variables, we used the categorical predictors cage compartment (random effect), experiment day (random effect) and butterfly species (fixed effect), and the continuous predictors absolute species density (consp-

Table 2 Results obtained in the cage experiment.

Conspecific density m^{-2}		Conspecific proportion		Number of pairs		Average mating proportion		<i>n</i>
<i>L. sinapis</i>	<i>L. juvernica</i>	<i>L. sinapis</i>	<i>L. juvernica</i>	<i>L. sinapis</i>	<i>L. juvernica</i>	<i>L. sinapis</i>	<i>L. juvernica</i>	
0.0083	0.0417	0.167	0.833	1	5	0.17	0.58	12
0.0417	0.0083	0.833	0.167	5	1	0.51	0	13
0.0250	0.0250	0.5	0.5	3	3	0.21	0.41	8
0.0083	–	1	–	1	0	0.67	–	6
–	0.0083	–	1	0	1	–	1	6

cific pairs m^{-2} ; fixed effect) and relative species density (number of conspecific individuals/total number of con- and heterospecific individuals in the cage compartment; fixed effect). To further look for possible nonlinearity (on logit scale) in the dependence on relative species density, we centred this covariate and allowed for linear, quadratic and cubic terms in the centred covariate. For the random effects, we also allowed for random slopes for the relative species density covariate, in addition to the random intercepts. We report the statistical significance of the fixed-effect terms in the selected lmer model.

We also verify these tests of statistical significance by fitting the parameters in the selected model using MCMCGLMM. There has been much discussion about the validity of P -values for fixed effects in mixed-effect models, and the tests of significance of fixed-effect parameters in Bayesian MCMC approach are often considered to be more reliable, which is why we use this approach to complement to the lmer model fitting (although in our case, there were no discrepancies between the conclusions from the lmer and the Bayesian approaches; for the latter, we used the P_{MCMC} P -values provided in the output of the MCMCGLMM function). For the Bayesian analyses, we assigned prior distributions for the random effects using suggestions in the documentation accompanying the MCMCGLMM software. We selected the parameters for the MCMC simulations (such as burn-in, thinning interval and the number of iterations) to achieve good mixing and verified this by examining autocorrelations from the simulated posterior distributions. See Data S1 for input data files and output from the statistical analysis, including information about Bayesian confidence intervals and effective sample sizes.

Results

Favourable weather conditions coincided with butterfly availability on 13 occasions in 2008 and 2009. In total, about 450 individual butterflies (including replacement individuals for escapees) of the two species participated in the experiments and were randomly assigned to one of the daily treatments of either being in the local minority (1 pair of one species: 5 pairs of the other species), in the local majority (5 : 1), in similar proportions as their sexual competitor (3 : 3) or alone in the cage without having to face heterospecific courtships (1 : 0). Only conspecific matings were observed. A spermatophore was found in all of the dissected females that had been observed to mate, whereas none of the females that were denoted as 'non-mated' had spermatophores in their *bursa copulatrix*.

There were large fitness costs of being in the minority. On average, across both species, 83% of females (Table 2) mated when alone in the cage with a conspecific male (1 : 0). In comparison, in only 8% of the

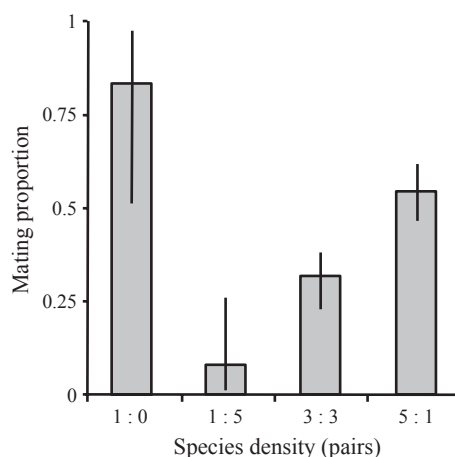


Fig. 1 Female mating success in relation to the species densities in a trial (conspecific pairs : pairs of the other species). Data are combined for *Leptidea sinapis* and *Leptidea juvernica* and are given as the proportion of females that mated in a trial together with 95% confidence intervals.

cases when the cage included a single conspecific couple and five couples of the other species (1 : 5), the male and female in the minority mated over the course of a trial (Table 2), whereas 32% of females in the treatment of three pairs of each species (3 : 3), and 54% of females in a five-to-one majority (5 : 1) mated (Table 2; Fig. 1). The statistical analysis showed that the mating success, measured as the probability of mating/day of a focal female, was strongly dependent on the relative species density, but was not statistically significantly affected by the absolute density of conspecific butterflies in the cage or by the species of the female. The AIC model selection procedure led to a model with only relative density and species as fixed effects (relative density: $z = 3.63$; $P < 0.001$; species: $z = -1.59$; $P = 0.11$) and random intercepts for experiment day and cage compartment. The Bayesian MCMC analysis of the selected model confirmed the tests of significance of the fixed effects (relative density: $P_{\text{MCMC}} < 0.001$; species: $P_{\text{MCMC}} = 0.06$). For all details on the statistical modelling and the subsequent model selection, see Data S1.

Discussion

The proportion of females that mated was strongly affected by the relative abundance of the species, but we did not find any additional influence of conspecific density. The importance of relative density is seen most clearly in a comparison between the 1 : 0 and 1 : 5 conditions (Fig. 1), with a striking drop in female mating success when heterospecifics were present. On the other hand, our data do not support the idea that the female mating probability would be primarily determined by the joint density of con- and heterospecific males in the cage,

because the mating proportions differed between the 1 : 5, the 3 : 3 and the 5 : 1 conditions, for which the overall male density is the same. Also, a comparison of these conditions shows that females were not more inclined to assess male quality and be more choosy under high conspecific male-density conditions (see e.g. Kokko & Mappes, 2005; Kokko & Rankin, 2006), because the mating probability was higher in 3 : 3 than in 1 : 5, and even higher in 5 : 1 (Fig. 1). Instead, our data show that there was a lower female mating probability for the species in the minority. In nature, a substantial proportion of a female *Leptidea*'s time budget is constrained by male courtship (con- or heterospecific), and females flying in local hotspots can be courted by up to ten males per hour (average 2.4 males/h; Berger *et al.*, 2012a). Thus, the densities experienced in our cage experiment probably did not exaggerate the fitness costs of being in the local minority. Therefore, our results support the idea that the time females spend being courted by heterospecific males can reduce the mating success of females of the species in the local minority.

In insects with a short expected lifespan, there are strong benefits of early reproduction (Berger *et al.*, 2012b), and the fitness cost of not finding suitable mates is predicted to be highest at low densities and to be a potential cause of population extinction (Bessa-Gomes *et al.*, 2004). Here, the heterospecific courtships impose additional minority costs on the rare species, as the relative density, rather than the absolute density, affected the female mating probability. In general, females are predicted to bear a higher cost than males of heterospecific sexual interactions. Post-mating costs are predicted to reinforce female mating preferences and select for assortative mating (Butlin, 1987; Howard, 1993; Liou & Price, 1994; Arnold *et al.*, 1996; Servedio & Noor, 2003). Premating costs, such as the ones we detected in *L. sinapis* and *L. juvernica*, could well be higher for females than for males (Parker & Partridge, 1998; Barry & Kokko, 2010), although it is not known to what extent this holds for *Leptidea*. At any rate, a possible evolutionary outcome of heterospecific reproductive interference is competitive exclusion through habitat separation (Hochkirch *et al.*, 2007). Previous studies on other butterflies have shown that unwanted male attention can force females into utilizing nonoptimal habitats for egg laying (Odendaal *et al.*, 1989; Baguette *et al.*, 1998). For *L. sinapis*, a recent study shows that also already mated females can spend a substantial proportion of their time constrained in (con- and heterospecific) male courtships in nature, and that male courtship has a negative effect on female fecundity measured over the short term (hours–days), but that this effect is likely to diminish over the course of the female lifespan (Berger *et al.*, 2012a).

Niche breadth has traditionally been studied at a population or species level, but a population's niche is

likely to be the sum of individuals with different niche requirements (Bolnick *et al.*, 2003). In the case of *L. sinapis* and *L. juvernica*, this would mean that selection from heterospecific courtships acts on individual variation in habitat preference and favours individuals that prefer habitats that maximize the chances of meeting conspecific partners. The habitat preferences of *L. sinapis* and *L. juvernica* have been experimentally tested in a Swedish population in a recapture experiment of laboratory-reared butterflies that were released either in a forest habitat or onto an adjacent meadow (Friberg *et al.*, 2008c). The results from this experiment showed that whereas the *L. juvernica* individuals quite soon settled in the meadow habitats, some *L. sinapis* butterflies crossed the forest–meadow border on several occasions, whereas others were recaptured again and again in their release habitats (Friberg *et al.*, 2008c). These data show both the innate (genetic) nature of the habitat utilization difference and the potential for within-species genetic variation in habitat preference among individuals for selection to act upon.

The minority effects detected in this study could be one potential driver behind the mosaic pattern of niche separation reported from different European populations of *L. sinapis* and *L. juvernica*. Under such a scenario, the direction of niche separation likely depends on the species that first populates an area. This could happen in two ways. Either the first colonizer settles only in the best possible habitat, with the result that these habitats/areas are no longer suitable for the second invader, or the first colonizer spreads into all suitable habitats, whereas the second invader is forced into specializing to a certain core area or habitat. The second alternative seems to be the most relevant explanation for the *Leptidea* complex, as the few cases when a species is alone in an area are characterized by habitat generalism (see Table 1 and Data S1).

Hence, we suggest a scenario in which the first colonizer fills up all suitable habitats (Fig. 2, t_0 – t_1), and the second invader is forced to specialize (Fig. 2, t_2 – t_3), if the cost of being rare is too large everywhere except in a core population with the highest relative conspecific density. The species in the local minority, on which selection from the heterospecific courtships is strongest, will then evolve towards habitat specialism, as individuals that leave the core population are likely to be strongly selected against due to intense sexual interactions with the closely related, more common species. This in turn would give rise to a dynamic landscape with local habitat patches being either sources or sinks depending on the local densities and the locally shaped habitat preferences of the different competitors. In a larger perspective, these dynamics will probably turn into a geographical mosaic of niche utilization similar to the one experienced by the sibling species of the *Leptidea* genus. Evolutionary effects of colonization order have previously been suggested to affect patterns

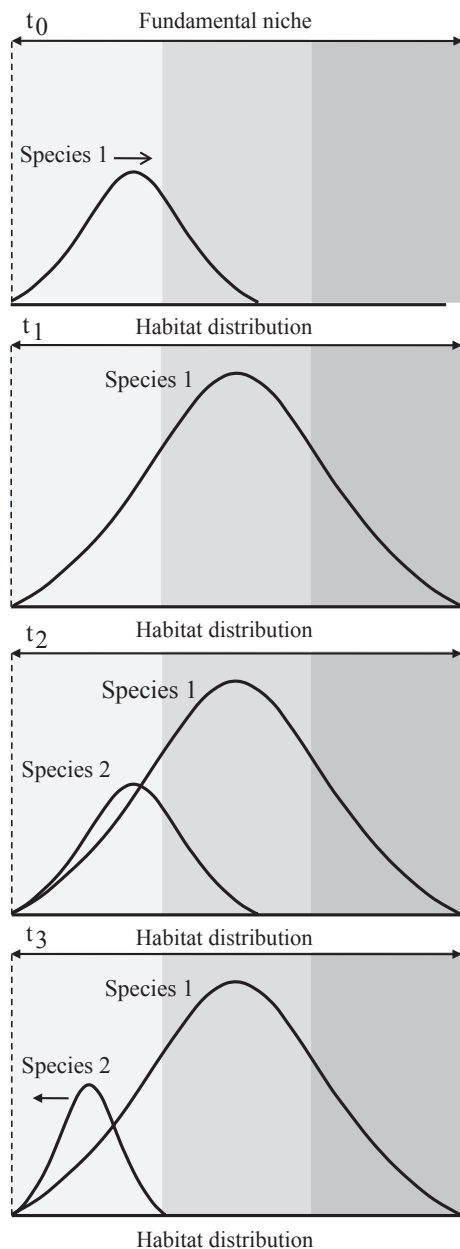


Fig. 2 The evolution of niche separation as proposed in this study. The first colonizer (species 1) enters an area at time zero (t_0) and spreads into all potential habitats (t_1). The second colonizer arrives in the area (t_2) and instead of spreading into the same area as species 1, selection favours habitat specialization in this species (t_3) due to the cost of being in the local minority inflicted by heterospecific interactions with species 1.

of community assembly (Price & Morin, 2004), as well as diversification rates in bacteria (Fukami *et al.*, 2007), and potentially also in macroorganisms (Seehausen, 2007). Future studies of the post-glaciation dispersal routes of the different *Leptidea* species and populations,

as well as studies that experimentally assess the habitat niche preferences of different European populations of *L. sinapis* and *L. juvernica*, are warranted to determine the possibility for colonization order to affect also the direction of niche separation and the evolution of generalization and specialization.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Additional background to the study system, the full dataset, and detailed information of the statistical models.

Figure S1 A *Leptidea sinapis* male (right) courting a conspecific female (left). Photo: Christer Wiklund.

Figure S2 (a) The two cages used in the study, (b) a view of the setup inside one of the cage compartments and (c) a *L. sinapis* male courting a *L. juvernica* female during the experiment. Photo: Magne Friberg.

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