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Niche separation in space and time between two sympatric sister species—a case of ecological pleiotropy

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Abstract We investigate the niche separation in space and time between the Palearctic sister species *Leptidea sinapis* and *L. reali* (Lepidoptera, Pieridae) in central Sweden. Using field sampling, we show that *L. reali* is a habitat specialist confined to meadows, whereas *L. sinapis* is a habitat generalist also inhabiting forests. This difference in habitat utilization was corroborated by experimental release of laboratory-reared *L. sinapis* and *L. reali* in two adjacent forest and meadow habitats during their natural flight period; virtually all recaptured *L. reali* that were released in the forest were later caught in the meadow, whereas *L. sinapis* shifted equally often from meadow to forest as in the opposite direction. In the field, both species fly in May–June, but *L. reali* appears on average a week earlier in spring and has a substantial second generation in July, whereas *L. sinapis* is practically univoltine. When overwintered pupae were incubated under identical conditions in the laboratory, females did, however, not differ in phenology, and *L. sinapis* males actually emerged earlier than *L. reali* males. When larvae were reared at 23°C on the host plant *Lotus corniculatus* at a range of daylengths, both species produced a substantial proportion of directly developing individuals at an 18.5 h daylength or longer. When reared at 23°C and a 22 h daylength, *L. reali* showed an overall higher propensity to develop directly than *L. sinapis* on plant species originating from both the meadow and the forest habitat. Both *Leptidea* species showed a lower propensity to enter direct development on forest associated plants than on meadow associated plants. Hence, we suggest that the difference in phenology and voltinism between

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L. sinapis and *L. reali* is largely the result of environmentally implemented ecological pleiotropic effects caused by the between-species difference in habitat preference.

Keywords Temporal and spatial niche-partitioning · Geographic mosaic · Secondary contact · Diapause · Direct development · Host plants

Introduction

During the last decades a growing body of evidence has assigned an important role for ecology in speciation and species maintenance (Losos et al. 1997; Feder and Filchak 1999; Rundle et al. 2000; Schluter, 2000; Hollander et al. 2005). In secondary contact zones between recently separated species ecological character displacement driven by interspecific competition is likely to continue the species divergence into less overlapping niches (Schluter 2000).

One such niche specialization is to utilize a habitat that is not occupied by interspecific competitors (Schlyter and Anderbrant 1993; Waugh and Weimerskirch 2003; Bearzi 2005; Boughman et al. 2005). Sympatric species might also dissociate along the temporal scale (Griffiths et al. 1991; Kronfeld-Schor and Dayan 2003), and so escape the negative impact of interspecific competition. Temporal niche partitioning is typically present on a diurnal scale, separating the peaks of activity of the coexisting competitors into different parts of the day (Kotler et al. 1993; Albrecht and Gotelli 2001, Gutman and Dayan 2005). In plants many closely related taxa differ in flowering time (Ellis et al. 2006; Levin 2006), while only a few zoological studies show such seasonal isolation, and many of these examples deal with more distantly related competitors (Bjelke et al. 2005; Dixon et al. 2005). Thus, temporal niche partitioning might lead to niche separation between species with similar habitat demands, although it has historically been regarded as far less common than separation in habitat or food preferences (Schoener 1974). The recent resurge of interest in the role for ecology in speciation has led to a renaissance for niche biology (Rundle and Schluter 2004). Hence, the significance and generality of ecological speciation needs to be further investigated, as well as the mechanisms that maintain the reproductive isolation between closely related species at an early stage of separation.

In 1988 Réal discovered that the former well-known pierid butterfly species *L. sinapis* was in fact composed of two largely sympatric species, and after some initial controversy there is now consensus that *L. sinapis* and *L. reali* are two separate *bona fide* species (Lorković 1993; Martin et al. 2003). Although the spring generations of *L. sinapis* and *L. reali* are morphologically virtually identical as far as wing coloration and wing patterns are concerned, they can easily be distinguished through genital preparation, male *L. reali* having a longer penis than male *L. sinapis* and the females of *L. reali* having a correspondingly longer ductus bursae than female *L. sinapis* (Lorković 1993; Mazel 2005), or DNA-sequencing (Martin et al. 2003).

The sister species (Martin et al. 2003) are spread all over Europe (Mazel 2002; Mazel and Eitschberger 2003), but collection studies from France, Spain and the Czech Republic show that the two species only rarely coexist in the same areas (Beneš et al. 2003; Amiet 2004; Vila et al. 2003). *L. sinapis* and *L. reali* appear to

have partitioned their habitat niches differently in different parts of Europe. In the Czech Republic and Slovakia, *L. sinapis* is rarer and restricted to warm areas of low elevations, while *L. reali* is widespread (Beneš et al. 2003). Moreover, the two species tend not to co-occur and *L. sinapis* is a habitat specialist occurring solely in warm and dry lowland habitats characterized by early-successional vegetation, whereas *L. reali* is a generalist occurring in these habitats as well as in damp meadows, bogs, fens and also mesic grasslands and woodland edges. In France, Amiet (2004) reported that *L. sinapis* and *L. reali* co-occur in only 23% of the sampled sites, supporting the view that the two species typically partition their niches. However, in France *L. sinapis* is the generalist and occurs from the lowest to the highest elevations and also includes several legumes as host plants, whereas *L. reali* is a specialist confined to habitats at high elevations and appears to be monophagous on *Lathyrus pratensis*. In Catalonia and Andorra, the two species partition their niches in a similar way with *L. reali* being a high-elevation specialist and *L. sinapis* being a generalist occurring in habitats from low to high elevations (Vila et al. 2003). Less is known about habitat partitioning of the two species in Croatia, but Lorković (1993) explicitly stated that here *L. reali* is most frequent on moist meadows but also inhabits mountainous habitats.

In principle, two scenarios can be envisaged how habitat niche partitioning is effectuated in different zones of secondary contact; either two interacting species have well-defined different niches that are repetitively implemented, or niche partitioning and character displacement are enacted de novo independently creating a geographic mosaic in which the basis for geographic variation is the local context where the evolving interactions occur (Thompson 2005). Recently, the importance of such geographic mosaics has highlighted the importance of large-scale geographic perspectives in studies of co-evolution. Independent interactions between species pairs are sometimes stable in different contact zones on a wide spatial scale, but might also vary depending on the local selection regime and the local genetic variation in the co-evolving species (Thompson 2005). Hence, when studying niche separation between closely related species the basis for geographic variation may well be the local context where the evolving interactions occur, a hypothesis that underlines the importance of studying interacting species over a large geographical range.

In this paper, we use observational field studies as well as laboratory and field experiments to study the on-going niche separation in space, time and voltinism between *Leptidea sinapis* and *L. reali* in south central Sweden. By studying niche separation on a local scale we aim to gain understanding of the processes that underlie the geographic mosaic of niche separation in other European zones of secondary contact.

Materials and methods

Study species

In central Sweden *L. sinapis* and *L. reali* have overlapping flight periods and the first generations fly from mid-May until early July (Eliasson et al. 2005). During the summer flight period from late July until late August a smaller number of directly developing butterflies are present. The two species are often found in the same areas

in meadow-fragmented forest landscapes (Eliasson et al. 2005). In Sweden, as well as in France and Germany, both species are oligophagous on legumes (Fabaceae) such as *Lathyrus pratensis*, *L. linifolius*, *Vicia cracca*, and *Lotus corniculatus* (Wiklund 1977b; Freese and Fiedler 2002; Amiet, 2004). Interestingly, males of both species court conspecific as well as heterospecific females both in the field (Friberg and Wiklund unpublished data) and in flight cages in the laboratory (Freese and Fiedler 2002), but the females exclusively accept conspecific mates (Freese and Fiedler 2002). Male courtship can last up to 30 min (Wiklund 1977a) and most likely involves energetic as well as opportunity costs for the displaying male. The lack of male species recognition, the close to identical morphology and the overlapping ecology indicate that the speciation is fairly recent and make the *Leptidea* species pair a good candidate for studies of niche separation at an early stage after speciation.

Species determination

All individuals used as founders of the laboratory populations used in this study were determined to species using both DNA-sequencing and genital preparations by NW and JK, respectively. Species were determined blind with respect to method used, i.e. neither determiner knew the supposed identity of the individual based on the other method. Genital preparations were made using standard methods. Butterflies that were species-determined using DNA-techniques were sequenced for one or two mitochondrial genes: the ND1-gene and the CO1-gene. The DNA was extracted from frozen or dried butterflies using QIAgen's DNEasy extraction kit. For each of the specimens ca. 730 bp of the NADH-dehydrogenase subunit one gene (ND1) was sequenced. Primers were taken from Martin et al. (2003). All PCRs were performed in a 20 μl reaction volume. The cycling profile for ND1 was 94°C for 2 min, then 30 cycles of 94°C for 30 s, 42°C for 30 s and 70°C for 1 min and a final 10 min extension at 70°C. The ND-forward PCR primer was also used for sequencing, which was done with a Beckman-Coulter CEQ8000 capillary sequencer. The resulting chromatogram was interpreted using the program BioEdit (Hall 1999) and the sequences were aligned by eye. In cases when the ND1 gene did not amplify, 320 bp of the cyto-chrome oxidase subunit one gene (COI) was sequenced, using primers LCO (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and k699 (5′-AGG AGG ATA AAC AGT TCA CC-3′). The cycling process for CO1 was 95°C for 5 min, 35 cycles of 94°C for 30 s, 47°C for 30 s, 72°C for 1 min and a final extension period of 72°C for 10 min. The LCO PCR-primer was also used for sequencing. ND1 sequences were compared to those from Martin et al. (2003) (GenBank Accession numbers AF485916 and AF485917 for *L. sinapis* and AF485914 and AF485915 for *L. reali*). Several individuals sequenced for ND1 were also sequenced for COI to compare variation in the two gene fragments.

Habitat preferences, phenology and voltinism of *L. sinapis* and *L. reali*

Field sampling

In 2005 at Riala (located 50 km north of Stockholm, 59°30′ latitude), a total of 84 butterflies were captured in a forest habitat and 55 were captured in an adjacent

meadow habitat between the 2nd of June and the 15th of July, to detect potential differences in habitat preference and phenology of the two species. In order to assess whether *L. sinapis* and *L. reali* have a second generation two localities were visited on every sunny day between the 20th of July and the 16th of August of 2005, both Riala and another sympatric population at Kronängen (located 100 km south of Stockholm, 59°0′ latitude)—a site with a similar meadow/forest structure as Riala. The time effort was equal for sampling butterflies in the meadow and forest habitat, so that when two of us were sampling at the same time one person was sampling in the forest and the other in the meadow habitat, and on days when only one person was sampling, butterflies were collected alternatively in the meadow and the forest during 1-h bouts. Butterflies were species determined using genital preparations.

Habitat preferences

Field experiment

The Riala forest was dominated by the trees *Pinus sylvestris* and *Picea abies* with occasional 1–3 m tall *Betula alba* growing in light gaps and in rocky areas where the soil layer was thin. *Pteridium aquilinum* was dominant among plants in the 1 m layer. The most common grasses were *Melica nutans*, *Deschampsia caespitosa* and *Dactylis glomerata*, and the most common *Leptidea* host plants were *Lathyrus linifolius*, *L. vernus* and *Vicia sylvatica*. The meadow, which in parts had been planted with young *Picea abies* in the year 2000 (now reaching a height of ca. 1 m), was dominated by the large herbs *Anthriscus silvestris* and *Angelica silvestris* with the grasses *Elytrigia repens*, *Phleum pratense*, *Alopecurus pratensis* and *Dactylis glomarata* growing abundantly, and also harboured the potential host plants *L. pratensis* and *Vicia cracca* in great abundance with *Lotus corniculatus* growing less abundantly in the drier parts of the meadow. Field observations of egg-laying females of *Leptidea* sp. have shown that butterflies utilize all the six legume host plants described above, even though *L. pratensis*, *L. linifolius*, *L. corniculatus* and *V. cracca* seem to be the most frequently utilized host plants in nature (Wiklund 1977b; Friberg and Wiklund unpublished data).

On three occasions, the 13th of June, the 20th of June and the 27th of June 2005, a total of 180 laboratory-reared, individually marked *L. reali* and 136 *L. sinapis* were released at Riala, a locality where both species flew abundantly at the time. Half of the butterflies of each species and sex were released in the forest and the remaining butterflies were released in a meadow immediately adjacent to the forest, in both cases 20 m from the boundary between the two habitats so that a shift from one habitat to the other could be easily accomplished for the butterflies.

The field site was visited every sunny day between the 13th of June and the 15th of July. All visible *Leptidea* butterflies were captured and their individual identity noted as well as the habitat in which they were recaptured. Thereafter the butterflies were re-released.

Phenology outdoors and at constant temperature

Nineteen females of *Leptidea* sp. were collected during May and June in the year of 2003 from Riala and Kronängen, and allowed to lay eggs on *L. pratensis* in cages

under laboratory conditions. Eggs and larvae were reared in climate cabinets under diapause-inducing conditions (16 h daylength, 23°C) until pupation, and 99 *L. sinapis* and 151 *L. reali* pupae were kept outdoors during the winter (22nd of August, 2003, until next spring). Larvae of both species pupated during a 2-week period in August 2003. The date of pupation per se is, however, of little relevance for the phenology, since pupal development is synchronized during winter and only post-diapause conditions affect the emergence date in spring (Wiklund and Solbreck 1982). On the 22nd of April 2004, 50 pupae of each species were transferred to a room maintained at 23°C and a 16 h daylength to assess innate potential differences in phenology between the species. The remaining pupae eclosed outdoors and were examined for emerging butterflies on a daily basis. All butterflies were sexed upon emergence and the date of emergence was noted.

Induction of diapause/direct development

(a) In relation to daylength

To experimentally test the possibility of divergent genetic photoperiodic responses between the species, 291 eggs from 12 laboratory-reared *L. reali* females and 422 eggs from 11 laboratory-reared *L. sinapis* females were divided among five climate cabinets (*Termaks Series KB8000L*), maintained at 23°C, and constant daylengths at 17:30, 18:00, 18:30, 19:00, and 19:30, respectively. All larvae were reared on *L. corniculatus*, a common host plant of both *L. sinapis* and *L. reali* (Lorković 1993; Freese and Fiedler, 2002) that was supplied ad libitum. The number of butterflies that entered direct development (without diapause) in the different climate cabinets was noted.

(b) In relation to host plant

In late summer/autumn 2005 and in a replicate in spring 2006 we tested whether the larval host plant influenced induction of diapause/direct development by rearing between 29 and 85 larvae of both species on each of six different host plants, *L. linifolius*, *L. pratensis*, *L. vernus*, *L. corniculatus*, *V. cracca* or *V. sylvatica*. All larvae were reared in climate cabinets (*Termaks Series KB8000L*) at a constant temperature of 23°C at a 22:00 h daylength. Larvae were reared in pairs in 0.5 l jars, and put in jars individually as soon as they reached pupation. Host plants were supplied ad libitum.

Statistic analysis

The collection data and the phenology experiments described above were analysed using General Linear Models (GLM) in Statistica 7.1 (StatSoft 2005). In cases when data did not show homogenous variances in the test groups according to Bartlett tests, data were transformed to meet the test assumptions. In all models concerning phenology we used species affiliation and sex as fixed factors, and the results were analysed using ANOVA (type III).

The binomially distributed data on diapause/direct development in five different light regimes were analysed using a logistic regression (GLZ) with logit as link

function in the statistical software R 2.2.1 for Windows (R Foundation for Statistical Computing).

When testing the propensity to enter direct development on plants associated with either the meadow or the forest habitat we used a mixed model nested ANOVA (type III) in Statistica 7.1 (StatSoft 2005), with replicate, butterfly species and habitat as fixed factors, host plant as a random factor nested within habitat, and the proportion of butterflies that entered direct development on the different plants as response value.

Results

Species determination

Species determinations using DNA sequences and genital morphology were 100% congruent. *L. sinapis* had distinct mtDNA haplotypes compared to *L. reali* for both the ND1 and the COI gene fragments. In the ND1 gene fragment, six sites (out of 730) were fixed for species-specific bases. In the COI gene fragment, five sites (out of 326) were fixed for species-specific bases (Table 1). Both sequences showed some intraspecific variation, but this did not confuse the species identifications and will be studied in more detail in the future. Unique haplotypes have been submitted to GenBank (*L. sinapis*: bankit787832 DQ387045; accession numbers will be made available later).

Habitat preferences

In the meadow habitat at Riala during June and early July in 2005, we collected 30 *L. reali* and 25 *L. sinapis*, whereas three *L. reali* and 81 *L. sinapis* were collected in the adjacent forest habitat during the same time period. This difference in habitat preference between the two species is statistically significant (Fisher exact $P < 0.001$; Fig. 1a). In the release-recapture experiment 84 of the 180 *L. reali* (47%) and 53 of the 136 *L. sinapis* (39%) that were released were later recaptured at least once. Forty-three of the *L. reali* individuals released in the forest were later recaptured on the meadow while only four individuals released on the meadow dispersed in the opposite direction. By contrast, 14 *L. sinapis* individuals released in the forest were recaptured on the meadow and 24 *L. sinapis* individuals shifted habitat from the meadow into the forest. Hence, both field sampling and the release-recapture experiment strongly indicate that *L. reali* prefers the meadow habitat, whereas *L. sinapis* commonly utilizes both meadow and forest habitats.

Table 1 Diagnostic differences in *L. sinapis* and *L. reali* COI-sequences used in this study

Species/position	3	60	72	97	132	147	193
Leptidea sinapis	C	R	T	G	C	A	T
Leptidea reali	T	A	C	A	T	G	C

Position numbers are based on sequence of *L. sinapis* (GenBank Accession number DQ387045). R = A or G
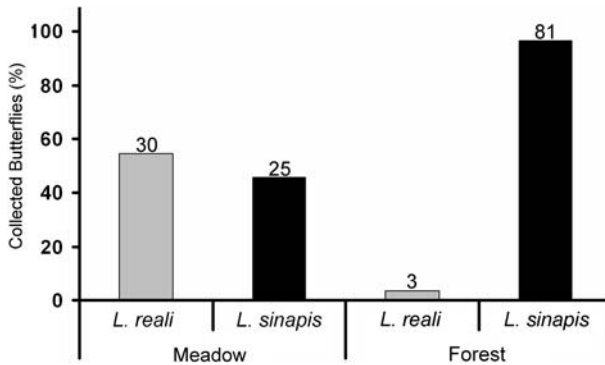
Fig. 1 The distribution of *L. sinapis and L. reali* in meadow and forest habitats presented as the proportion of the total number of butterflies captured in each habitat

The average time between release and last recapture was between 6 and 8 days for both *L. sinapis* and *L. reali*, although some individual males and females of both species survived for 3 weeks or longer (*L. reali*$_{\male\male}$ = 7.7 days ± (SD) 4.6 days, max = 23 days; *L. reali*$_{\female\female}$ = 7.4 days ± 5.3 days, max = 21 days; *L. sinapis*$_{\male\male}$ = 6.9 days ± 7.1 days, max = 26 days; *L. sinapis*$_{\female\female}$ = 6.0 days ± 4.9 days, max = 26 days).

Phenology in the field

The average collection date was earlier for male *L. reali* than male *L. sinapis*, and this pattern also held for females. Furthermore, *L. reali* males ($n = 23$) were caught significantly earlier than *L. reali* females ($n = 10$) but no difference was found in the collection date between male ($n = 70$) and female ($n = 36$) *L. sinapis* (GLM: *species*: $F_{1,135} = 33.54$, $P < 0.001$; *sex*: $F_{1,135} = 7.59$, $P = 0.0067$; *species *sex*: $F_{1,135} = 6.04$, $P = 0.015$; Tukeys HSD test between sexes, within species: $P_{L.sinapis} = 0.98$, $P_{L.reali} = 0.016$). The average collection date was for *L. reali* males the 8th of June ± (SD) 7 days, for *L. reali* females the 20th of June ± 11 days, for *L. sinapis* males the 27th of June ± 11 days, and for *L. sinapis* females the 28th of June ± 12 days (Fig. 2a). Hence, although both species were on the wing from the beginning of June until mid-July, *L. reali* appears earlier in the season than *L. sinapis*.

Phenology outdoors and at constant temperature

Among the hibernating pupae that were kept outdoors under a natural daylength and temperature conditions from 22nd of April until emergence, males emerged significantly earlier than females in both species, and males of *L. sinapis* ($n = 17$) emerged earlier than males of *L. reali* ($n = 47$), but no difference in emergence date was found between the females of *L. sinapis* ($n = 32$) and *L. reali* ($n = 54$). Data were log-transformed to meet the assumption of homogenous variances (GLM: *species*: $F_{1,146} = 10.03$, $P = 0.0019$; *sex*: $F_{1,146} = 73.03$, $P < 0.001$; *species*sex*: $F_{1,146} = 9.23$, $P = 0.0028$; Tukeys HSD test: $P_{between\ males} < 0.001$, $P_{between\ females} = 0.99$). The average date of emergence (±SD) was the 11th of May ± 2 days for

Fig. 2 (**a**) The average collection date (±SD) of each species and sex, during the collection period between late May and early July 2005. (**b**) The average emergence date (±SD) for pupae eclosing in natural outdoor temperature and daylength conditions. (**c**) The average number of days (±SD) until emergence when pupae were transferred to a constant temperature of 23°C on April 22, after spending 8 months outdoors
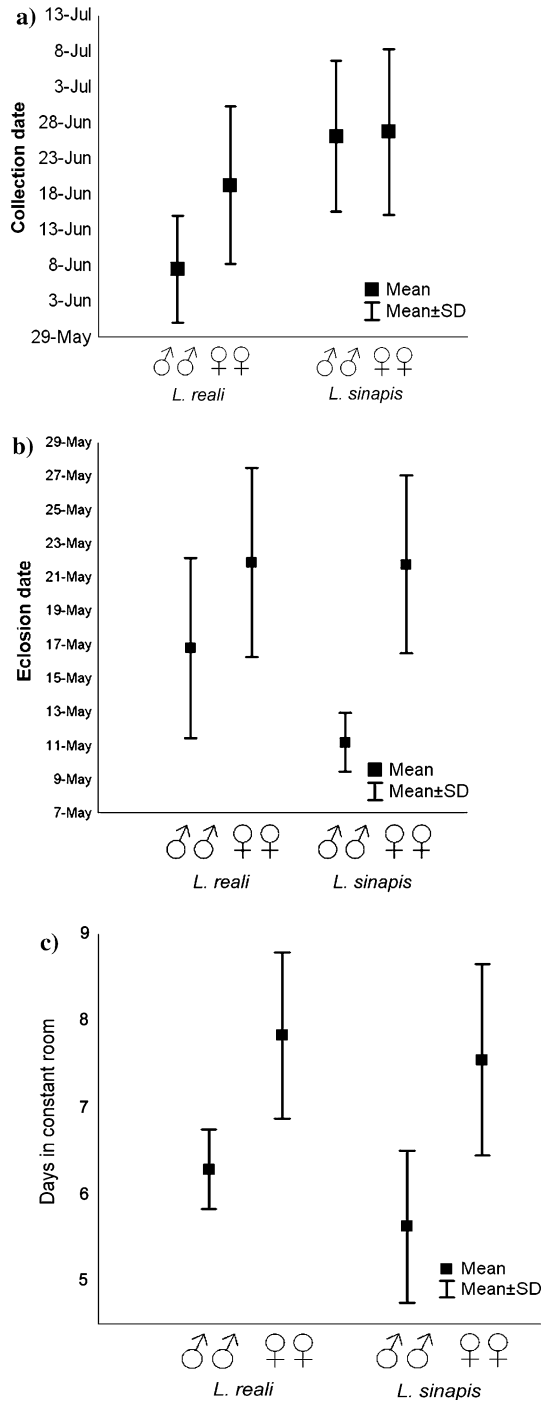
L. sinapis males, the 22nd of May ± 5 days for *L. sinapis* females, the 17th of May ± 5 days for *L. reali* males and the 22th of May ± 6 days for *L. reali* females (Fig. 2b).

When hibernating pupae were transferred to a constant temperature of 23°C, on the 22nd of April (i.e. after 8 months under outdoor conditions) males of both species emerged earlier than their respective females. *L. sinapis* males ($n = 24$) also emerged earlier than *L. reali* males ($n = 25$), but there was no difference between the females of *L. sinapis* ($n = 24$) and *L. reali* ($n = 22$). Data were log-transformed to meet the assumption of homogeneous variances (GLM: *species*: $F_{1,91} = 7.91$, $P = 0.006$; *sex*: $F_{1,91} = 88.93$, $P < 0.001$; *species*sex*: $F_{1,91} = 1.78$, $P = 0.19$; Tukeys HSD test $P_{between\ males} = 0.019$, $P_{between\ females} = 0.73$). The average emergence day after incubation for females of both species was between 7 and 8 days ± 1 day (SD), whereas male *L. sinapis* emerged after 5 to 6 days ± 1 day, and *L. reali* males after 6 days ± 0.5 days (Fig. 2c).

Voltinism in the field

Field sampling between the 20th of July and the 16th of August yielded not a single butterfly from Riala, but 53 butterflies, 52 *L. reali* and one *L. sinapis*, from the more southern locality of Kronängen. Hence, *L. sinapis* appears to be largely univoltine in central Sweden, whereas *L. reali* abruptly changed from partial bivoltinism to univoltinism over a distance corresponding to 30′, or approximately 100 km in a south-to-north direction.

Induction of diapause/direct development

(a) In relation to daylength

When reared at 23°C the proportion of *L. reali* that entered direct development increased from 0 to 100% within a 90 min change in daylength, from 18:00 to 19:30 h (Fig. 3). *L. sinapis* responded very similarly to increasing daylength, although the propensity to enter direct development differed statistically from that of *L. reali* (GLZ: $\chi^2_4 = 9.51$; $P_{species*day\ length} = 0.0496$), due to a higher *L. sinapis* propensity to enter direct development at intermediate daylengths (Fig. 3). It is also noteworthy that for *L. sinapis* the proportion of butterflies that entered direct development did not go from 0 to 100%; there was always a small proportion of individuals that developed directly at short daylengths, and also a small proportion that entered diapause development at the very long daylengths (Table 2).

(b) In relation to host plant habitat

The survival of both species varied between different plants. Typically the larvae had difficulties in establishing during the first larval instar on all plants and *L. reali* suffered exceptional problems on *V. sylvatica*, with only 13 out of 66 larvae surviving the first instar, and only two larvae surviving until pupation in replicate one, and 4 survivors in replicate two (Table 3).

L. reali had a higher propensity to enter direct development on all host plants in both replicates except on *V. sylvatica* in the first replicate where the only two survivors entered diapause development (Table 4). Both *L. reali* and *L. sinapis*

Fig. 3 Propensity to enter direct development among *L. sinapis* and *L. reali* larvae in terms of the percent (and 95% confidence intervals) of individuals that developed directly when reared on *Lotus corniculatus* at 23°C at five different daylengths from 17:30 to 19:30 h
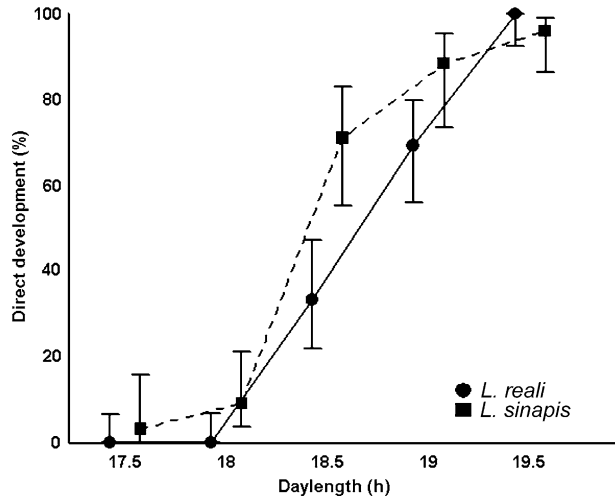
Table 2 Larval establishment and survival to pupation in five different photoperiods

Species	Daylength	Number of eggs	Larval establishment	Larval survival after establishment
L. reali	17:30	61	93% (57)	98% (56)
	18:00	59	88% (52)	100% (52)
	18:30	60	88% (53)	96% (51)
	19:00	60	92% (55)	100% (55)
	19:30	51	92% (47)	98% (46)
L. sinapis	17:30	85	58% (49)	65% (32)
	18:00	84	64% (54)	81% (44)
	18:30	82	54% (44)	86% (38)
	19:00	82	54% (44)	77% (34)
	19:30	89	67% (60)	82% (49)

Table 3 Larval establishment and survival to pupation on six different host plants (both replicates added)

Species	Habitat association	Host plant	Number of eggs	Larval establishment	Larval survival after establishment
L. reali	Meadow	*Lotus corniculatus*	64	77% (49)	94% (46)
		Lathyrus pratensis	98	51% (50)	92% (46)
		Vicia cracca	83	61% (51)	90% (46)
	Forest	*Lathyrus linifolius*	75	77% (58)	90% (52)
		Lathyrus vernus	59	49% (29)	79% (23)
		Vicia sylvatica	66	20% (13)	46% (6)
L. sinapis	Meadow	*Lotus corniculatus*	77	40% (31)	84% (26)
		Lathyrus pratensis	137	52% (71)	90% (64)
		Vicia cracca	68	54% (37)	97% (36)
	Forest	*Lathyrus linifolius*	67	76% (51)	67% (34)
		Lathyrus vernus	66	58% (38)	87% (33)
		Vicia sylvatica	61	67% (41)	73% (30)

Table 4 Number of *L. sinapis* and *L. reali* larvae that entered diapause- or direct development, respectively, on different host plants

Species	Host plant habitat	Host plant	Replicate 1			Replicate 2			Total		
			Diapause dev.	Direct dev.	Direct dev. %	Diapause dev.	Direct dev.	Direct dev. %	Diapause dev.	Direct dev.	Direct dev. %
L. reali	Meadow	*Lotus corniculatus*	0	24	100%	1	21	95%	1	45	98%
		Vicia cracca	2	21	91%	1	22	95%	3	43	93%
		Lathyrus pratensis	2	20	90%	4	20	83%	6	40	87%
	Forest	*Lathyrus linifolius*	7	24	77%	2	18	90%	9	42	82%
		Lathyrus vernus	6	9	60%	2	6	75%	8	15	65%
		Vicia sylvatica	2	0	0%	0	4	100%	2	4	67%
L. sinapis	Meadow	*Lotus corniculatus*	3	11	79%	0	12	100%	3	23	88%
		Vicia cracca	7	9	56%	12	8	40%	19	17	47%
		Lathyrus pratensis	14	17	55%	20	13	39%	34	30	47%
	Forest	*Lathyrus linifolius*	13	1	7%	14	6	30%	27	7	21%
		Lathyrus vernus	19	0	0%	8	6	43%	27	6	18%
		Vicia sylvatica	10	10	0%	8	2	20%	18	12	40%

exhibited a higher propensity to develop directly when reared on plants growing in meadow habitats, viz. *L. corniculatus*, *L. pratensis*, and *V. cracca* (Mossberg et al. 1992), whereas both butterfly species showed a lower propensity to develop directly when reared on plants growing in forest habitats, viz. *L. linifolius*, *L. vernus* and *V. sylvatica* (Direct development (%): *L. sinapis* $\bar{u}_{\text{meadow plants}} = 61\%$ ($\pm$SD 24%), $\bar{u}_{\text{forest plants}} = 26\%$ ($\pm$12%); *L. reali* $\bar{u}_{\text{meadow plants}} = 93\%$ ($\pm$5%), $\bar{u}_{\text{forest plants}} = 71\%$ ($\pm$10%); Table 5, Fig. 4).

Discussion

### Ecological pleiotropy: habitat preference, phenology and voltinism

Swedish *Leptidea* butterflies have different habitat preferences. *L. reali* tends to be more of a specialist strongly preferring the meadow habitat, while *L. sinapis* exhibits

Table 5 Results from a mixed model nested ANOVA showing the propensity to enter direct development as dependent on the two butterfly species, the different host plant habitats (forest/meadow) and the two replicates

Factor	Effect	SS	df	MS	F	P
Intercept	Fixed	9.10	1	9.10	202.53	0.00014
Replicate (R)	Fixed	0.088	1	0.088	1.49	0.25
Butterfly species (BS)	Fixed	0.81	1	0.81	15.54	0.017
Host plant habitat (HPH)	Fixed	0.58	1	0.58	12.92	0.023
Host plant species (in HPH)	Random	0.18	4	0.045	0.87	0.55
BS*HPH	Fixed	0.0178	1	0.018	0.34	0.59
HPS (BS*HPH)	Random	0.21	4	0.052	0.88	0.51
Error		0.65	11	0.059		

Host plant habitat was tested over host plant nested in host plant habitat. Interactions involving replicate were not significant and have been removed from the table

Fig. 4 Propensity to enter direct development on forest- and meadow associated host plants for *L. sinapis* and *L. reali*. Shown are means and 95% confidence intervals
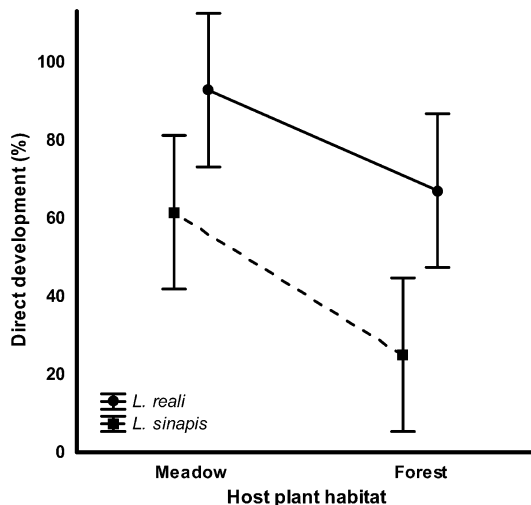
wider habitat preferences, dwelling both in forest and meadow habitats. In spring *L. reali* emerges earlier than *L. sinapis* in nature, but no such difference was found regarding emergence date in the controlled experiments. This implies that the difference in phenology is not directly genetically implemented but rather an ecological pleiotropic effect caused by the difference in habitat preference.

Recently, the role of ecological pleiotropy has gained considerable interest (Jiggins et al. 2001; Dres and Mallet 2002). Essentially, this phenomenon means that a small genetic change affecting one niche dimension might cause non-genetic ecological pleiotropic effects that result in substantial between-species differences in multidimensional niches space. Such an ecological pleiotropic link has earlier been shown between mimicry traits and mate choice, where the fitness of certain mimicry morphs varies between areas and populations. Assortative mating is therefore favoured within each population both by chance as a pleiotropic effect due to the rarity of other phenotypes and by selection due to decreased hybrid fitness from stabilizing selection (Jiggins et al. 2001; Dres and Mallet 2002).

In the *Leptidea* species pair, the earlier emergence of *L. reali* in the field appears to be such a pleiotropic effect, since pupae hibernating on the meadows are likely to eclose earlier in spring due to the more sun-exposed circumstances there, whereas pupae located in the forest emerge somewhat later in spring. Given that the location of the pupae mirrors the habitat preferences of the adults, it should be expected that *L. reali* has a relatively shorter early emergence period associated with the meadow habitat, whereas *L. sinapis* exhibits a prolonged emergence period associated both with the early meadow and late forest habitats. The difference in average flight period was not affected by differences in longevity, since no differences in lifespan were detected between either species or sex during the release-recapture experiment, and both species were recaptured in similar proportions, which makes it unlikely that the two species differ in propensity or ability to disperse.

The difference in habitat utilization also appears to induce the differences in protandry pattern that we observed between the two species. Males of both *L. sinapis* and *L. reali* eclosed earlier than their respective females in the controlled laboratory experiments, but only male *L. reali* were protandrous in the field. It is highly probable that all *L. reali* individuals belonged to the meadow cohort, i.e. the first eclosing butterflies, while *L. sinapis* butterflies emerged both during the meadow eclosion period and during the later forest eclosion period. Therefore, *L. sinapis* males are most likely protandrous also in the field, but within each cohort, so that any protandry pattern disappears once the *L. sinapis* butterflies that have eclosed in the forest mix with conspecifics eclosed from hibernation on the meadow.

Furthermore, the differences in habitat preferences and phenology could also influence the observed difference in voltinism. At Kronängen, a sympatric population with a similar habitat structure (forest/meadow) as Riala, a substantial second generation of butterflies was discovered in July/August, but no second generation butterflies were found at Riala. Hence, the northern limit for direct development seems to be located in south central Sweden, between the latitudes of Kronängen and Riala, even though a larger number of populations need to be sampled to exclude potential effects of local climate on the possibilities to develop into a summer generation at the two sites. Moreover, it is obvious that *L. reali* produced a substantial second generation, whereas *L. sinapis* at best produced a very small one. This difference does not seem to be caused by fixed differences in the photoperiodic responses, and the proportion of *L. sinapis* butterflies that entered direct

development in relation to daylength actually exceeded the *L. reali* proportion at intermediate daylengths under controlled experimental conditions (Fig. 3). Also this pattern could conceivably be explained if larvae of *L. reali* are located in more sun-exposed positions and experience higher temperatures than those of *L. sinapis*, under the hypothesis that higher temperature increases the propensity for direct development at a given daylength (Danilevsky 1965; Tauber et al. 1986).

Besides daylength, the larval host plant affected the propensity to develop directly in both species. *L. reali* entered direct development in a higher proportion than *L. sinapis* on all host plants, but both species experienced longer development times on the host plants most closely associated with the forest habitat and also entered direct development in a lower proportion on the forest plants. Hence, the observation that *L. reali* produced a much more numerous second generation than *L. sinapis* is only partly explained by genetic between-species differences in propensity to enter direct development. In addition, the between-species difference in habitat utilization causes an ecological pleiotropic effect that exaggerates the difference in proportion of butterflies that enter direct development, since it is highly probable that a higher proportion of *L. reali* than *L. sinapis* is using meadow associated host plants such as *L. pratensis*, *V. cracca* and *L. corniculatus*.

Since Danilevsky's (1965) discovery that daylength and temperature are of overriding importance for the induction of diapause/direct development, most studies have focused on these stimuli (Wiklund et al. 1991, 1992; Fischer and Fiedler 2001). However, some studies have shown an effect of the host plant species in the propensity to enter direct development in other multivoltine species. In these cases, the host plants have been either quite distantly related (Wedell et al. 1997) or plants with obvious differences in their nutritional value (Hunter and McNeil 1997). However, larvae of *L. sinapis* and *L. reali* exclusively feed on different species within one plant family, and therefore our finding adds a novel aspect to the issue of how/if host plants influence the induction of diapause/direct development. Further studies are needed to investigate the role of host plants for the possibility to have multiple generations per year, and more specifically to investigate whether the induction of diapause/direct development is influenced by host plant species per se, or mediated through variation in the possibilities of a rapid development on different host plants.

The geographic mosaic of specialist/generalist habitat use

The niche separation into one habitat specialist and one habitat generalist species is to our knowledge quite rare. Typically both interacting species specialize into different niches (Boughman et al. 2005). Unfortunately, the habitat usage of the ancestor of *L. sinapis* and *L. reali* is unknown, and therefore we do not know whether *L. sinapis* has widened its niche to also include the forest habitat, or if the *L. reali* preference for meadows is the result of habitat specialization. Earlier studies have shown that generalists evolve into specialists and vice versa, at least in terms of host plant utilization (Janz et al. 2001). It is not possible to draw any further conclusions from our data, and a resolved phylogeny is needed to unravel the direction of the niche separation.

The picture that emerges above with *L. sinapis* and *L. reali* changing specialist and generalist roles with respect to habitat utilization suggests an explanation in line with the geographic mosaic scenario. However, it is also necessary to consider the

possibility that habitat specialization or generalization may not only be dependent on the actual habitats as such, but also on secondary effects of other, micro-scale differences such as host plant choice. Experiments both in Germany (Freese and Fiedler 2002) and France (Amiet 2004) have revealed that there is a geographically consistent pattern how the females rank the host plants during egg-laying; *L. sinapis* prefers to oviposit on *L. corniculatus*, whereas *L. reali* prefers *L. pratensis*. It is conceivable that differences in host plant preference might also predict the distribution of the two butterfly species in addition to for example altitude or forest cover. To make a valid inference with respect to the role of host plant preference for habitat specialization, a key study would be to investigate the host plant preferences of *L. sinapis* and *L. reali* in the Czech Republic. It is also necessary to more closely study egg-laying behaviour in the field in order to further understand the dissimilarities in niche separation in different parts of Europe.

What is the driving force behind the niche separation between *L. sinapis* and *L. reali* is still an open question; the niches of the two species might have diverged due to genetic drift or have been forced apart by selective forces that minimize interspecific competition and heterospecific courtship interactions. Regardless, the occurrence of ecological pleiotropy that deepens the niche differences between *L. sinapis* and *L. reali* might explain the geographic mosaic of local niche adaptations among and between the two species in different European populations. Hence, a small genetic difference may cause environmentally induced larger differences in the ecological niches and so be instrumental in implementing a geographic mosaic in which different local populations rapidly adapt to assume different character states or niches. Earlier, such environmentally, or ecologically, implemented pleiotropies have been reported mostly from studies on assortative mate choice (Jiggins et al. 2001; Dres and Mallet 2002, McKinnon et al. 2004; Hollander et al. 2005), and further research is needed to determine the consequences of ecological pleiotropy on speciation, reinforcement and character displacement, interspecific competition and niche plasticity.

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