

## Experimental evidence for specific distinctness of the two wood white butterfly taxa, *Leptidea sinapis* and *L. reali* (Pieridae)

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**Summary.** In mating experiments in a flight cage females, and to a lesser extent the males, of *Leptidea sinapis* und *L. reali* discriminated during mate choice. As a consequence only intraspecific matings occurred within these two morphologically defined taxa. The possibility of speciation through sexual selection and female choice is discussed. The response of both *Leptidea* species towards four food plants (*Lotus corniculatus*, *Lathyrus pratensis*, *Vicia cracca*, *Medicago sativa*) was experimentally studied. Ovipositing females in choice tests showed significantly different preferences, with *L. reali* favouring *L. pratensis*, while *L. sinapis* preferably laid eggs on *L. corniculatus*. Both species largely rejected *M. sativa*. With regard to fitness parameters such as prepupal weight, developmental duration and growth rate, rank orders of the tested food plants were equal for both *Leptidea* species. *Lotus corniculatus* was the optimal host, followed by *Lathyrus pratensis* and *Vicia cracca*, with *Medicago sativa* being least favourable. Interspecific differences in life-history parameters were small. *L. reali* grew on average slightly larger, while *L. sinapis* had shorter development times and higher growth rates. The extent of protandry was 2 days in both *Leptidea* species. In food-choice tests fourth (= final) instar larvae of both *Leptidea* species preferred *L. corniculatus*; *M. sativa* was rarely chosen. Ranking of food plants in choice situations was similar in the two *Leptidea* species and matched their ranking with regard to larval fitness. Discrepancies between preference and performance occurred in *L. reali* (relative rank of *L. pratensis* versus *L. corniculatus*) and point towards an evolutionarily young, not yet fixed ecological differentiation between the two *Leptidea* species. Our experimental findings support the notion that *L. reali* and *L. sinapis* are true biospecies with ethological reproductive isolation, but only minimal differentiation with regard to ecology and life-history.

**Zusammenfassung.** In Verpaarungsversuchen in einem Flugkäfig diskriminierten vor allem die Weibchen, aber nur begrenzt die Männchen, von *Leptidea sinapis* und *L. reali* bei der Partnerwahl. Infolgedessen kam es ausschließlich zu intraspezifischen Kopulationen innerhalb der beiden morphologisch definierten Taxa. Die Möglichkeit der Aufspaltung beider Arten durch sexuelle Selektion und Weibchenwahl wird diskutiert. Die Reaktion beider *Leptidea*-Arten gegenüber 4 Fraßpflanzen (*Lotus corniculatus*, *Lathyrus pratensis*, *Vicia cracca*, *Medicago sativa*) wurde experimentell geprüft. Bei der Eiablage zeigten die Weibchen in Wahlversuchen signifikant unterschiedliche Präferenzhierarchien, wobei *L. reali* zugunsten von *L. pratensis* diskriminierte, während *L. sinapis* bevorzugt auf *L. corniculatus* ablegte. Beide Arten mieden *M. sativa* weitgehend. Für beide *Leptidea*-Arten galt dieselbe Rangfolge der Pflanzenarten in Bezug auf deren entwicklungsphysiologische Qualität (gemessen über Präpuppengewichte, Entwicklungsdauer und Wachstumsrate). *Lotus corniculatus* war die hochwertigste Pflanzenart, gefolgt von den relativ gleichwertigen Arten *Lathyrus pratensis* und *Vicia cracca* und an letzter Stelle *Medicago sativa*. Zwischenartliche Unterschiede in den Lebenszyklusparametern waren gering. *L. reali* wurde im Mittel etwas größer, *L. sinapis* dagegen zeigte kürzere und raschere Entwicklung. Das Ausmaß der Protandrie war mit 2 Tagen Entwicklungsdifferenz bei beiden *Leptidea*-Arten gleich. In Futterwahlversuchen wählten Raupen im vierten (= letzten) Larvalstadium beider *Leptidea*-Arten bevorzugt *L. corniculatus*, *M. sativa* wurde kaum angenommen. Die Rangfolge der Fraßpflanzen in Wahlsituationen galt für beide *Leptidea*-Arten gleichermaßen und stimmte mit der Rangfolge der ernährungsphysiologischen Eignung überein. Diskrepanzen zwischen Eiablagepräferenz und Eignung der Fraßpflanzen traten bei *L. reali* auf (relativer Rang von *L. pratensis* versus *L. corniculatus*) und deuten auf eine junge, noch nicht gefestigte ökologische Differenzierung der beiden *Leptidea*-Arten hin. Nach diesen experimentellen Befunden sind *L. reali* und *L. sinapis* echte Biospezies mit ethologischer Fortpflanzungsbarriere, aber nur geringfügig differenziert im Hinblick auf Ökologie und Lebenszyklus.

**Résumé.** Lors d'expériences d'accouplement en cage de vol, les femelles, et en moindre mesure les mâles, de *Leptidea sinapis* et de *L. reali* eurent une attitude discriminante lors du choix du partenaire. En conséquence, seulement des accouplements intraspécifiques eurent lieu parmi ces deux taxons morphologiquement définis. La possibilité d'une spéciation par sélection sexuelle et par choix par la femelle est discutée. La réponse des deux espèces de *Leptidea* envers quatre plantes nourricières (*Lotus corniculatus*, *Lathyrus pratensis*, *Vicia cracca*, *Medicago sativa*) a été étudiée expérimentalement. Lors de la ponte durant des tests de choix, des différences significatives furent observées, *L. reali* favorisant *L. pratensis*

alors que *L. sinapis* pondait de préférence sur *L. corniculatus*. Les deux espèces rejetèrent largement *M. sativa*. Eu égard aux paramètres de fitness tels que le poids prépupal, la durée de développement et le degré de croissance, les ordres par degré pour les plantes nourricières utilisées lors de ces expériences furent les mêmes pour les deux espèces. *Lotus corniculatus* était la plante-hôte optimale, suivie de *Lathyrus pratensis* et de *Vicia cracca*, *Medicago sativa* étant la moins appropriée. Les différences interspécifiques au niveau des paramètres biologiques étaient faibles. *L. reali* atteignit une taille moyenne légèrement supérieure, alors que *L. sinapis* avait une durée de développement inférieure et un degré de croissance plus important. La protérandrie était de 2 jours pour les deux espèces. Lors de tests de préférence nourricière, les chenilles au quatrième (et ultime) état des deux espèces de *Leptidea* préférèrent *L. corniculatus*; *M. sativa* était rarement retenue. La classification en ordre de préférence des plantes nourricières en situations de choix était similaire pour les deux espèces de *Leptidea* et correspondait à leur classification eu égard au fitness larvaire. Des oppositions entre préférence et performance étaient apparentes chez *L. reali* (rang relatif de *L. pratensis* par rapport à *L. corniculatus*) et semblent indiquer une différenciation très récente des deux espèces de *Leptidea* du point de vue évolutif, qui n'est pas encore fixée écologiquement. Nos observations expérimentales sont à l'appui de la notion que *L. sinapis* et *L. reali* sont de vraies bio-espèces reproductivement isolées du point de vue éthologique, mais ne manifestant qu'une différenciation minimale aux niveaux écologique et biologique.

**Key words.** Biospecies, ethological reproductive isolation, life-history, preference, performance, sibling species, sexual selection, female choice, hostplant relationships.

## Introduction

Though there is still no universal consensus about how to define a 'species' (Hey 2001), this category remains the central unit for many branches of biology such as phylogenetics or biodiversity research. Among zoologists the biospecies concept is often accepted as the operationally most useful one (e.g. Collins 1991, Luckow 1995). A biospecies is defined as a 'group of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups' (Mayr 1942). Although this concept has not gone unchallenged and many alternatives are still disputed in the literature (Hey 2001), its major advantage is that it allows for an objective experimental testing of species boundaries. The two major approaches to test for specific distinctness of two putative entities are (1) to measure gene flow between natural populations or (2) to attempt crossings under controlled environmental conditions. Such tests then reveal at which positions in the continuum between complete reproductive isolation and free gene flow two entities are situated.

In practical taxonomy, however, recognition of species is frequently based on phenotypic differentiation alone. This 'morphospecies concept' is at odds with evolutionary theory, since static morphological entities cannot evolve *per se*. Morphospecies are categories subjectively defined by human observers on the grounds of 'similarities' and are *a priori* not natural entities. Yet, since phenotypic differentiation frequently is based on genotypic divergence, the morphospecies concept remains a useful surrogate for species as true biological entities as long as relevant information about reproductive isolation is non-existent.

In recognizing species boundaries among taxonomically complex species groups (such as sibling species), phenotypic evidence almost always predates, and mostly even stimulates, research on potential reproductive isolation. Thereby, the morphologically based hypothesis on the existence of two (or more) species is tested in the framework of the biospecies concept.

Even in well studied taxa and regions, such as butterflies in central Europe, occasionally new phenotypic entities continue to be discovered that are then described as new



species. The wood white butterflies of the genus *Leptidea* provide one of the most interesting examples in the past 15 years. A number of ecological and behavioural studies had dealt with '*Leptidea sinapis* (Linnaeus, 1758) sensu lato' (e.g. Wiklund 1977a, 1977b, Wiklund & Solbreck 1982, Warren 1984, 1985; Warren *et al.* 1986). Yet, in 1988 Réal (1988) recognized on the grounds of genitalia studies that this well known taxon might rather comprise a sibling species complex: *L. sinapis* s. str. and a newly separated taxon, *L. reali* Reissinger, 1989 (= *L. lorkovicii* Réal, 1988). Females (Réal 1988) as well as males (Lorković. 1993) can be diagnosed by means of genitalia measurements. External characteristics such as wing colours are less suitable, since they vary strongly within both taxa in a similar way. Only extreme wing phenotypes appear to allow for a reliable discrimination (Mazel 2000, 2001a).

The status of *L. reali* as a distinct species was immediately accepted in many publications including identification guides (e.g. Tolman & Lewington 1998). Faunistic studies revealed that *L. sinapis* and *L. reali* co-occur over wide areas of Europe (Mazel 2000, 2001a, 2001b). Only few authors (Lorković. 1993; Kudrna 1998) remained more sceptical and called for detailed research to substantiate the hypothesis of specific distinctness between *L. reali* and *L. sinapis*. One reason for this wide and rapid acceptance of the specific status of *L. reali* might be found in the high value currently placed on genitalia characters in Lepidopteran taxonomy. Disregarding the fact that genitalia, like any other morphological feature, exhibit phenotypic plasticity and intraspecific variation (Goulson 1993; Monti *et al.* 2001), differences in genitalia morphology are readily accepted as indicators for the existence of true biospecies. Underlying this conception is the lock-and-key hypothesis (e.g. Kullenberg 1947; Shapiro & Porter 1989), according to which genitalia differences were to provide prezygotic reproductive isolation barriers. However, support for this hypothesis is scant (Mikkola 1992; Sota & Kubota 1998), and there is increasing evidence that in many cases genitalic differentiation is more related to sexual selection and cryptic female choice (Eberhard 1993; Arnqvist 1998) rather than maintaining reproductive isolation.

Apart from confirmations of the genitalic differentiation, critical studies on the status of both *Leptidea* forms as biospecies were lacking thus far (e.g. Lorković. 1993). Similarly, published indications of some ecological divergence between both forms are all derived from anecdotal observational evidence, without controlling for any confounding variables and without any statistical evaluation of the results. For example, differential preferences of females for egg-laying substrates have been postulated (e.g. Lorković. 1993; Kristal & Nässig 1996), with *L. reali* preferentially ovipositing on *Lathyrus* spp. while *L. sinapis* should lay eggs more freely on *Lotus corniculatus* or *Vicia cracca*.

Therefore, the aim of the present study is two-fold: (1) to assess by means of mating experiments under controlled conditions whether *L. reali* and *L. sinapis* butterflies recognize each other as distinct species and thus really avoid hybridization; and (2) to assess whether the two forms differ from each other with regard to egg-laying, food acceptance or larval performance on a range of hostplants that have been recorded to be utilized by the *L. sinapis* complex.

## Material and methods

**Egg-laying experiments.** Butterflies from both *Leptidea* forms were brought into the laboratory for egg-laying. Insects were sampled during the emergence of both generations (1<sup>st</sup> generation: May, 2<sup>nd</sup> generation: mid July to mid August) in the vicinity of Bayreuth and Würzburg (northern Bavaria).

To test for oviposition preferences, field-caught females were placed singly in glasses (2 litres) covered with gauze and lined with moist filter paper. A small vial with concentrated sucrose solution was added for nourishment. Glasses were placed in an environmental chamber (25 °C constant, L:D regime 18:6 h).

Each female was offered simultaneously three small bunches of oviposition substrates, viz. *Medicago sativa*, *Lathyrus pratensis*, and *Lotus corniculatus* in the first generation and *Lathyrus pratensis*, *Lotus corniculatus* and *Vicia cracca* (all Fabaceae) in the second generation. These plant species have been recorded frequently as hostplants (Thomas & Léwington 1991; Bink 1992; Ebert & Rennwald 1993) and were readily available in sufficient supply. Care was taken to only offer young foliage of each plant species in approximately equal amounts. Every day the bunches were exchanged and the numbers and placement of all eggs laid during the preceding day was noted. Egg-laying was followed for each experimental female until her death. For each female taxonomic identity was subsequently assessed by dissecting the genitalia. All these females could be unequivocally assigned to one of the two morphospecies using the genitalic characters described in the literature.

For statistical evaluations only females were considered who laid at least 10 eggs in captivity. For each individual the proportions of eggs laid on any available food plant species was calculated, taking her lifetime fecundity as 100%. These proportions were then compared between the two *Leptidea* species.

**Effects of food plants on performance and fitness.** Until hatching, eggs were kept in the same environmental chamber as the adult butterflies. Offspring of each female was kept separately throughout the entire development. Upon hatching, larvae were transferred in groups of two individuals into transparent plastic vials (250 ml) lined with moist filter paper. To circumvent diapause and standardize developmental conditions, caterpillars and pupae were placed in an environmental chamber under long day conditions (25 °C constant, L:D regime 18:6 h). Fresh food was supplied in excess every second day. During the fourth instar caterpillars were reared individually to avoid food competition.

We simultaneously reared offspring of the first generation of both species in no-choice tests on either *Lathyrus pratensis*, *Lotus corniculatus* or *Medicago sativa*, respectively. Offspring of the second generation received *Lathyrus pratensis*, *Lotus corniculatus* or *Vicia cracca*, respectively. Plants of the genus *Medicago* have rarely been recorded as *Leptidea* hostplants (e.g. Bink 1992). By including *M. sativa* we aimed to test how strongly larval performance was affected if larvae were forced to develop on this apparently less preferred hostplant species. Within each generation larvae were randomly assigned to the food treatments.

For each caterpillar the following parameters were recorded: duration of larval development, duration of the fourth larval instar, mass at the beginning of the fourth



larval instar, prepupal mass, duration of pupal phase. The relative growth rate in the final larval instar was calculated from these data as:  $RGR = [\ln(\text{prepupal weight}) - \ln(\text{initial weight})]/\text{duration of instar}$ .

Previous analyses have shown that growth rate (apart from body size and development time) should be treated as a life-history parameter in its own right and that the above version of calculating growth rates has a number of statistical advantages (see Nylin & Gotthard 1998; Fischer & Fiedler 2001). Weights were determined on an electronic Sartorius MC 210P balance to the nearest 0.1 mg. Since prepupae had to be removed from their girdle for weighing, the resulting pupae were later fixed using double-sided sticky tape. This procedure ensured safe metamorphosis of the great majority of individuals (> 95%).

**Food choice experiments.** For food choice experiments, fourth instar larvae of both species were placed individually in Petri dishes (12 cm diameter, height 1.5 cm) lined with moistened filter paper. Larvae for these tests were randomly chosen from the mass rearing which occurred on the two sets of three plant species each in the first and second generation (see above). All larvae were used for a test only once. The plant species on which a test larva had developed prior to the experiment was recorded as the factor 'Reared' for subsequent analysis, whereas the plant species chosen was noted as 'Selected'. Thus it was possible to account for inductions of preferences through earlier feeding experience. Each animal was then allowed to choose between three food plant species offered simultaneously in such a way that foliage of each plant species covered approximately one third of the area, while the centre of the Petri dish remained free. The test caterpillar was introduced in this centre with random orientation. The Petri dishes were then placed in the same environmental cabinet as the other larvae. After 24 h we recorded on which of the three plants the caterpillars were actually feeding.

Food choice tests were performed with the same plant combinations as the no-choice performance experiments. Larvae in spring and early summer were offered *Lathyrus pratensis*, *Lotus corniculatus* and *Medicago sativa*, while those in late summer were offered *Lathyrus pratensis*, *Lotus corniculatus* and *Vicia cracca*.

**Mating experiments.** To obtain intra- and possibly interspecific matings, a flight cage (2.4 × 1.2 × 1.2 m) covered with gauze was placed in a greenhouse at 30 °C and 60% relative humidity, illuminated by strong lamps that emitted a sufficient UV fraction. In this cage butterflies of both sexes and species could fly freely. Since all individuals were marked uniquely by numbers that had been maintained throughout their development and since all mother butterflies had been identified based on genitalic dissection after their death, the taxonomic identity of each butterfly individual in the flight cage was known with certainty. As food sources vials with sucrose solution and bunches with natural nectar sources (*L. corniculatus* flowers) were offered. In total, 30 to 60 butterflies were present at each observation in the cage whose mean age was 2.2 d (range: 0–10 d). Observations were conducted for 2–3 h/d on 11 d during emergence of the summer generation. Whenever courtship behaviour occurred, the following data were recorded: the individual numbers of the butterflies involved, duration of courtship sequences (measured with a stop watch), the female's receptiveness (indicated by

her bending of the abdomen towards the courting male or, alternatively, by her avoidance behaviour), and the incidence and duration of a resulting copulation.

**Statistical evaluation.** Results were evaluated using standard statistical procedures (Sachs 1997) as implemented in the package Statistica 6.0 (StatSoft 2001). Throughout the text, means are reported  $\pm$  one standard deviation. Test statistics and sample sizes are given for each type of comparison. Where multiple tests on the same data set had to be performed, we applied a sequential Bonferroni correction (Hochberg 1988) to maintain a table-wide significance threshold of  $p=0.05$ .

## Results

**Oviposition preferences.** Females of both species laid eggs on all offered food plants. Some eggs were even laid on non-plant substrates such as glass, filter paper or gauze. Numbers of eggs laid per female were low and did not differ between species (*L. reali*:  $37.2 \pm 31.8$  eggs (range 2–143,  $n=38$ ); *L. sinapis*:  $33.1 \pm 35.8$  eggs (range 4–129,  $n=18$ ); Mann-Whitney *U*-test:  $z=0.94$ ,  $p>0.34$ ; Fig. 1). Mean fecundity was thus similar to the value reported by Bink (1992) for '*L. sinapis sensu lato*', while maximum fecundity in our samples was much higher (up to 143 eggs). Eighteen *L. reali* and 10 *L. sinapis* females laid enough eggs to allow for a statistical evaluation. Among these, interspecific differences were noted only for egg-laying on *L. pratensis* (Mann-Whitney *U*-test:  $z=2.52$ ,  $p<0.01$ , significant after sequential Bonferroni correction). *L. reali* females laid a larger fraction of their egg-load on this plant species as compared to *L. sinapis* (Fig. 2).

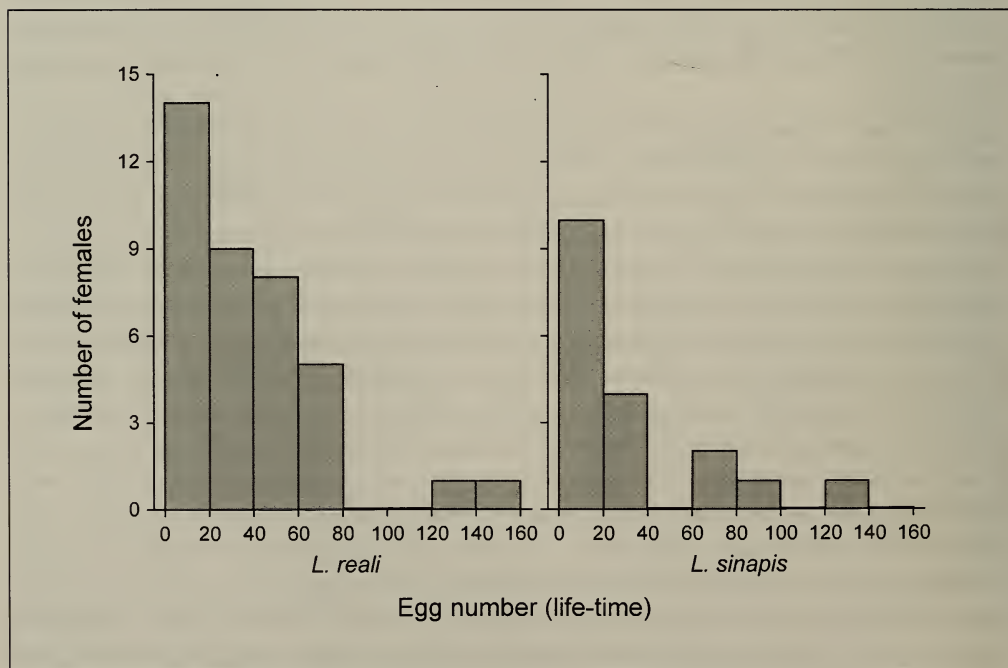
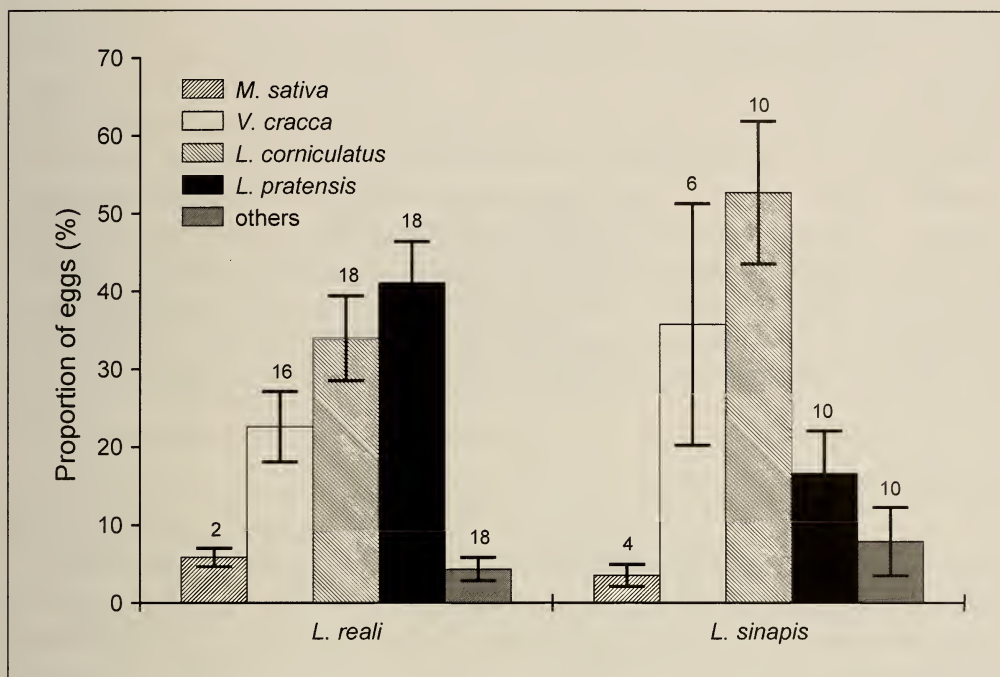


Figure 1. Lifetime fecundity of captive *Leptidea* females.



**Figure 2.** Proportion of eggs (means  $\pm$  1 S.D.) laid by captive females of *L. reali* and *L. sinapis* when offered a choice between various food plants. Numbers above columns refer to numbers of females in the tests that laid more than 10 eggs.

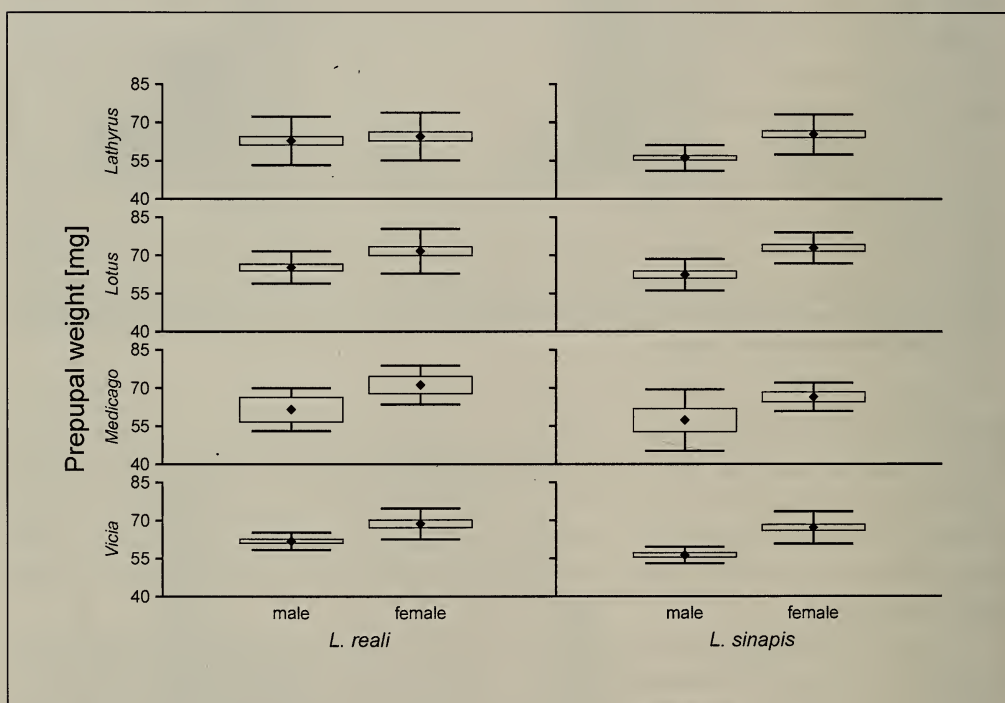
There was substantial individual variation in egg-laying preferences in both species. Nine *L. reali* females laid more than 50% of their egg-load on *L. pratensis*, five on *L. corniculatus* and two on *V. cracca*. For *L. sinapis*, the respective numbers were one (*L. pratensis*), five (*L. corniculatus*, including one female that laid all her eggs on this plant species) and two (*V. cracca*, including one female that laid all her eggs on this plant species).

A slightly different picture emerges if all eggs laid during the experiments are summed up. Of the 752 eggs laid by 25 *L. reali* females, *M. sativa* received 0.9%, *L. corniculatus* 33.9%, *L. pratensis* 40.4% and *V. cracca* 18.2% (5.2% were laid on non-host substrates). The respective figures for the 436 eggs laid by 15 *L. sinapis* females were: 1.6% eggs on *M. sativa*, 53.2% on *L. corniculatus*, 22.2% on *L. pratensis* and 16.3% on *V. cracca* (6.7% on non-host substrates). However, these cumulative data cannot be subjected to a statistical analysis since eggs laid by the same female cannot be treated as independent data points and individuals that happened to lay more eggs would be over-represented.

Collectively, the oviposition experiments revealed that *L. sinapis* and *L. reali* differ in their oviposition hierarchies, although individuals of both species may exhibit very different responses. For *L. reali*, the hierarchy was *L. pratensis*  $\geq$  *L. corniculatus* > *V. cracca* >> *M. sativa*, whereas for *L. sinapis* it was *L. corniculatus* > *V. cracca*  $\geq$  *L. pratensis* >> *M. sativa*.



Development and performance of early stages in no-choice experiments. Prepupal weights differed between sexes, with females generally being 10% larger than males (Fig. 3, Table 1). There was also a highly significant, albeit slight effect of food plant, with larvae reared on *L. corniculatus* achieving highest weights in both species and sexes, while the three other food plants were of equal quality as measured by prepupal weights. Finally, *L. reali* reached overall slightly (and significantly) higher weights ( $\delta$ :  $63.2 \pm 7.5$  mg;  $\eta$ :  $68.2 \pm 8.8$  mg) than *L. sinapis* ( $\delta$ :  $58.0 \pm 6.6$  mg;  $\eta$ :  $67.7 \pm 7.4$  mg) under identical rearing conditions. This effect was more pronounced in males, although statistically the species  $\times$  sex interaction just failed to reach significance. There was no species  $\times$  food interaction suggesting that performance of the two *Leptidea* species was not differentially affected by the food plant in no-choice situations.



**Figure 3.** Prepupal weights of *L. reali* and *L. sinapis*, according to sex and food plant, obtained under standardized climatic conditions (25°C constant, 16:8h L:D cycle). Filled diamonds: means; boxes:  $\pm 1$  SD, whiskers:  $\pm 1$  SE.

There were highly significant differences between species, sexes and food plants with regard to total development times (i.e. entire larval and pupal duration; Table 2, Fig. 4). Generally, females of both species took about 2 d longer to develop. Moreover, in both sexes *L. reali* required about 1 d longer than *L. sinapis* to reach maturity under identical environmental conditions (*L. reali*:  $\delta$ :  $26.2 \pm 2.7$  d (n=88);  $\eta$ :  $28.6 \pm 2.6$  d (n=88); *L. sinapis*:  $\delta$ :  $25.1 \pm 2.6$  (n=77);  $\eta$ :  $27.1 \pm 2.2$  d (n=91)). Developmental duration was shortest for both species and sexes when fed *L. corniculatus*, insects reared on *L.*



**Table 1.** Results of three-way ANOVA of prepupal weights of *L. sinapis* and *L. reali*, with species, sex and food plant as main factors. Significant effects printed in bold. d.f. = degrees of freedom.

	d.f.	Mean square	F	p
<b>Species</b>	<b>1</b>	<b>395.3</b>	<b>7.53</b>	<b>0.006</b>
<b>Sex</b>	<b>1</b>	<b>3081.6</b>	<b>58.68</b>	<b>&lt;0.0001</b>
<b>Food</b>	<b>3</b>	<b>604.0</b>	<b>11.50</b>	<b>&lt;0.0001</b>
Species × Sex	1	170.5	3.25	0.07
Species × Food	3	35.6	0.68	0.56
Sex × Food	3	65.2	1.24	0.29
Species × Sex × Food	3	33.6	0.64	0.59
Error	297	52.5		

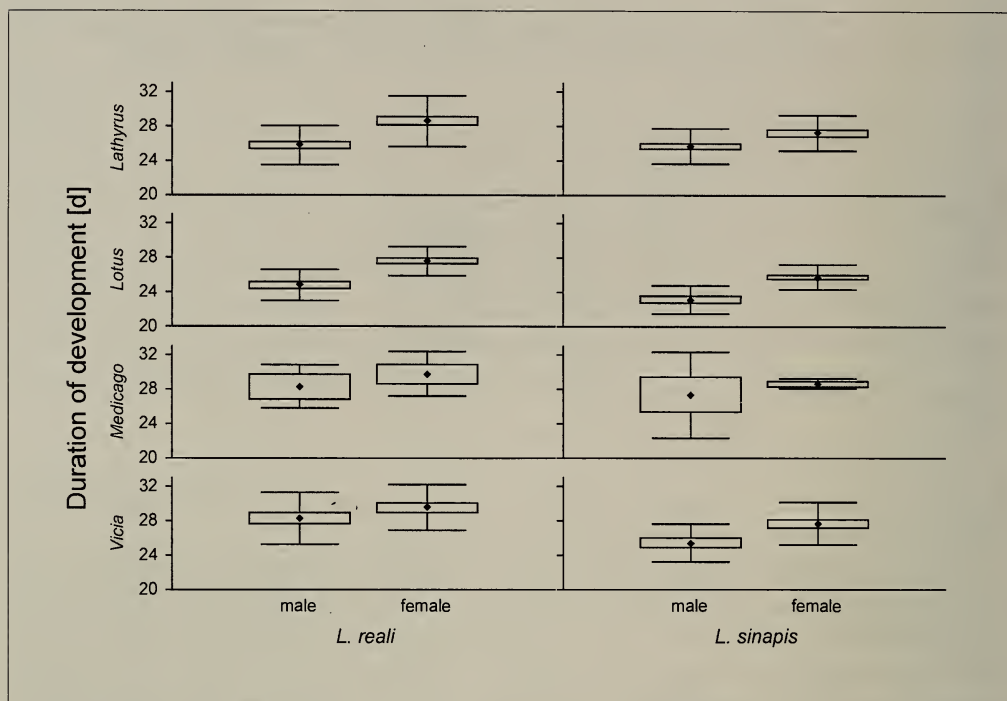
*pratensis* required 1–2 d longer, and rearing on *V. cracca* or *M. sativa* retarded development by about 2–4 d as compared to *L. corniculatus*.

Growth rates significantly differed between sexes (with males growing by about 6% more rapidly) as well as between species (*L. sinapis* having about 12.3% higher growth rate than *L. reali*; Table 3, Fig. 5). Food plant species did not affect growth rate. There was a significant, though weak species × food interaction which was due to the fact that *L. reali* grew more slowly on *V. cracca* and *L. pratensis*, whereas growth rates were almost equal across all food treatments in *L. sinapis*. Collectively, when integrating all three life-history parameters measured, larval performance was best on *L. corniculatus*, with only weak differences between the other three food plant species. Food choice by caterpillars.

A log-linear analysis (Table 5) of food choice frequencies (Table 4) was performed. Since the three-way interaction *Species* × *Reared* × *Selected* was not significantly different from zero, only two-way interactions were included in the search for an optimal model. This optimal model (goodness of fit excluding structural zeros: maximum-likelihood  $\chi^2_{12 \text{ df}} = 7.06$ ,  $p > 0.85$ ) revealed that feeding decisions of caterpillars were influenced by the plant species on which a caterpillar had initially fed (*Reared* × *Selected* interaction). For example, *V. cracca* was never chosen by larvae initially fed *L. corniculatus*. *L. corniculatus* was particularly often selected by larvae initially fed this same plant species or *L. pratensis*, whereas larvae initially reared on *V. cracca* or *M. sativa* did not prefer *L. pratensis* over *L. corniculatus*.

There was no significant *Species* × *Selected* interaction, i.e. both *Leptidea* species behaved similarly when given a choice between the food plants selected for experiments. Structural zeros (marked in Table 4) reflect that not all choice opportunities were possible to the larvae. Since experiments were conducted during two generations, with two different sets of plant species, neither decisions of *Vicia*-reared larvae towards *Medicago*, nor decisions of *Medicago*-fed larvae to *Vicia*, were possible.

Statistical significance of the factor *Reared* is biologically meaningless. This simply shows that the numbers of caterpillars taken from the various initial food plants differed (due to differential availability in our rearings). The factor *Selected* was highly

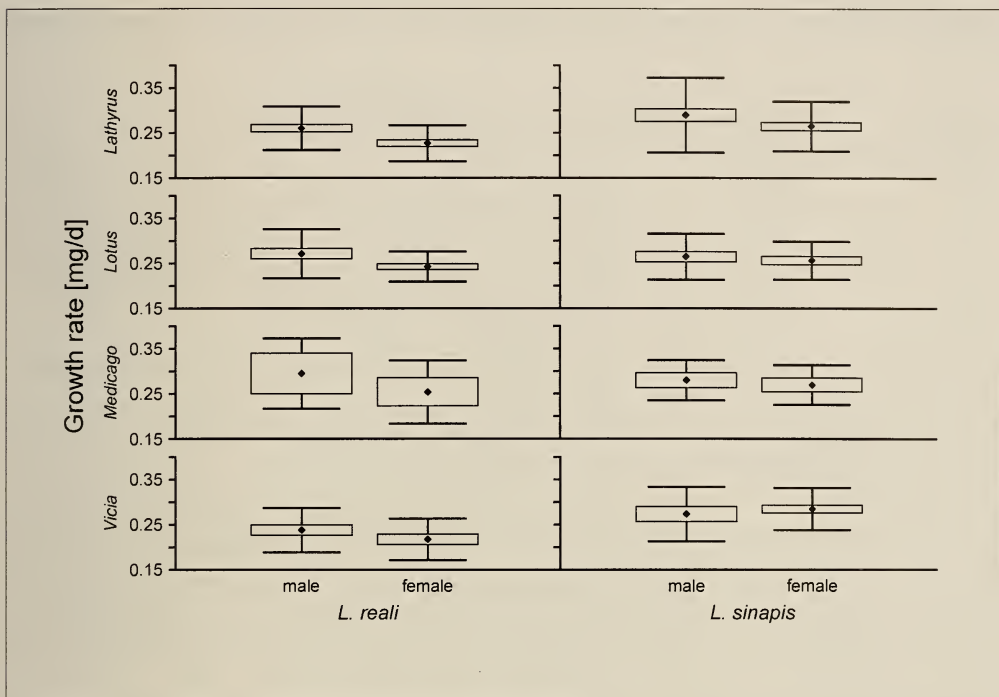


**Figure 4.** Duration of development (all larval instars plus pupal stage) of *L. reali* and *L. sinapis*, according to sex and food plant, obtained under standardized climatic conditions (25°C constant, 16:8h L:D cycle). Filled diamonds: means; boxes:  $\pm 1$  SD, whiskers:  $\pm 1$  SE.

**Table 2.** Results of three-way ANOVA of developmental times of *L. sinapis* and *L. reali*, with species, sex and food plant as main factors. Significant effects printed in bold. d.f. = degrees of freedom.

	d.f.	Mean square	<i>F</i>	p
<b>Species</b>	<b>1</b>	<b>91.2</b>	<b>16.95</b>	<b>&lt;0.0001</b>
<b>Sex</b>	<b>3</b>	<b>103.1</b>	<b>19.16</b>	<b>&lt;0.0001</b>
<b>Food</b>	<b>1</b>	<b>170.0</b>	<b>31.58</b>	<b>&lt;0.0001</b>
Species $\times$ Sex	3	13.3	2.48	0.061
Species $\times$ Food	1	0.2	0.04	0.85
Sex $\times$ Food	3	4.1	0.75	0.52
Species $\times$ Sex $\times$ Food	3	5.4	1.01	0.39
Error	328	5.4		

significant, indicating that the plant species offered differed strongly in their acceptability. A subsequent comparison of observed against expected choice frequencies (under the null hypothesis of equal choice of all four food plant species) revealed that both *Leptidea* species discriminated between plants (*L. sinapis*:  $\chi^2_{3df}=25.9$ ,  $p<0.0001$ ; *L. reali*:  $\chi^2_{3df}=30.1$ ,  $p<0.0001$ ). Identification of the plants that contributed to this unevenness in choice decisions showed that both *Leptidea* species chose *L. corniculatus* significantly more often than expected by chance (*L. sinapis*:  $\chi^2_{1df}=10.9$ ,  $p<0.001$ ; *L.*



**Figure 5.** Growth rate during fourth (= final) larval instar of *L. reali* and *L. sinapis*, according to sex and food plant, obtained under standardized climatic conditions (25°C constant, 16:8h L:D cycle). Filled diamonds: means; boxes:  $\pm 1$  SD, whiskers:  $\pm 1$  SE.

**Table 3.** Results of three-way ANOVA of growth rates of *L. sinapis* and *L. reali*, with species, sex and food plant as main factors. Significant effects printed in bold. d.f. = degrees of freedom.

	d.f.	Mean square	F	p
<b>Species</b>	<b>1</b>	<b>0.023</b>	<b>8.11</b>	<b>0.005</b>
<b>Sex</b>	<b>1</b>	<b>0.018</b>	<b>6.22</b>	<b>0.013</b>
Food	3	0.002	0.86	0.46
Species $\times$ Sex	1	0.006	2.21	0.13
<b>Species <math>\times</math> Food</b>	<b>3</b>	<b>0.009</b>	<b>3.15</b>	<b>0.025</b>
Sex $\times$ Food	3	0.002	0.79	0.50
Species $\times$ Sex $\times$ Food	3	0.0006	0.20	0.90
Error	297	0.003		

*reali*:  $\chi^2_{1df}=11.8$ ,  $p=0.0006$ ), whereas *M. sativa* was strongly discriminated against (*L. sinapis*:  $\chi^2_{1df}=18.1$ ,  $p<0.0001$ ; *L. reali*:  $\chi^2_{1df}=10.1$ ,  $p=0.0015$ ; all comparisons significant after sequential Bonferroni correction).

Collectively, these data demonstrate that in choice situations caterpillars of both *Leptidea* species select food plants in much the same way, the hierarchy being: *L. corniculatus* > *L. pratensis* = *V. cracca* >> *M. sativa*.



**Table 4.** Frequencies of food choice decisions, depending on the initial rearing plant. \*: structural zeros, i.e. this choice opportunity was not offered to the larvae.

Species	Initial rearing food plant	Selected plant after 24h	Frequency
<i>Leptidea reali</i>	<i>L. corniculatus</i>	<i>L. corniculatus</i>	11
		<i>L. pratensis</i>	8
		<i>V. cracca</i>	0
		<i>M. sativa</i>	0
	<i>L. pratensis</i>	<i>L. corniculatus</i>	32
		<i>L. pratensis</i>	17
		<i>V. cracca</i>	7
		<i>M. sativa</i>	1
	<i>V. cracca</i>	<i>L. corniculatus</i>	12
		<i>L. pratensis</i>	11
		<i>V. cracca</i>	10
		<i>M. sativa</i>	0*
	<i>M. sativa</i>	<i>L. corniculatus</i>	8
		<i>L. pratensis</i>	8
		<i>V. cracca</i>	0*
		<i>M. sativa</i>	1
<i>Leptidea sinapis</i>	<i>L. corniculatus</i>	<i>L. corniculatus</i>	18
		<i>L. pratensis</i>	10
		<i>V. cracca</i>	0
		<i>M. sativa</i>	1
	<i>L. pratensis</i>	<i>L. corniculatus</i>	29
		<i>L. pratensis</i>	20
		<i>V. cracca</i>	8
		<i>M. sativa</i>	4
	<i>V. cracca</i>	<i>L. corniculatus</i>	13
		<i>L. pratensis</i>	13
		<i>V. cracca</i>	4
		<i>M. sativa</i>	0*
	<i>M. sativa</i>	<i>L. corniculatus</i>	8
		<i>L. pratensis</i>	8
		<i>V. cracca</i>	0*
		<i>M. sativa</i>	1

**Mating experiments.** We observed a total of 158 courtship events in the flight cage equipped with butterflies of both species and sexes (Table 6). Males courted not only females of their own species, but did so also towards heterospecific females. However, there was a clear difference between both species, with *L. reali* males courting *L. sinapis* females disproportionally rarely, while *L. sinapis* males courted heterospecific females even more frequently than their conspecifics (Fisher's exact

**Table 5.** Log-linear analysis of decision frequencies in food choice tests. Significant effects printed in bold. d.f. = degrees of freedom.

	d.f.	partial $\chi^2$	partial p	marginal $\chi^2$	marginal p
Species	1	0.43	0.51	0.43	0.51
<b>Reared</b>	<b>3</b>	<b>54.3</b>	<b>&lt;0.0001</b>	<b>54.3</b>	<b>&lt;0.0001</b>
<b>Selected</b>	<b>3</b>	<b>156.0</b>	<b>&lt;0.0001</b>	<b>156.0</b>	<b>&lt;0.0001</b>
Species × Reared	3	1.41	0.70	1.77	0.62
Species × Selected	3	2.01	0.57	2.37	0.50
<b>Reared × Selected</b>	<b>9</b>	<b>22.2</b>	<b>0.008</b>	<b>22.6</b>	<b>0.007</b>

**Table 6.** Observed frequencies of intersexual interactions in a large flight cage.

Species of ♂	Species of ♀	courtship	♀ receptive	copula	♂ attempts copula, ♀ unreceptive
<i>L. reali</i>	<i>L. reali</i>	75	9	7	0
<i>L. sinapis</i>	<i>L. sinapis</i>	26	7	5	2
<i>L. reali</i>	<i>L. sinapis</i>	3	0	0	0
<i>L. sinapis</i>	<i>L. reali</i>	54	0	0	7
total intraspecific		101	16	12	2
total interspecific		57	0	0	7

test,  $p < 0.0001$ ). When courting, males settled down in front of a sitting female and rapidly moved around their extended proboscis. Intraspecific courting sequences lasted  $19.1 \pm 32.5$  s in *L. reali* ( $n=53$ ) and  $20.2 \pm 37.6$  s in *L. sinapis* ( $n=20$ ; *U*-test:  $z=0.75$ ,  $p>0.4$ ). Interspecific courtships were of similar length as intraspecific ones (male *L. reali* courting female *L. sinapis* ( $n=2$ ):  $19.4 \pm 33.7$  s; male *L. sinapis* courting female *L. reali* ( $n=42$ ):  $23.5 \pm 30.9$  s; *U*-tests for intra- versus interspecific courtships by male *L. reali*:  $z=0.29$ ,  $p>0.77$ ; by male *L. sinapis*:  $z=1.76$ ,  $p=0.087$ ).

In 16 cases females signalled receptiveness by bending their abdomen towards the courting male. This response exclusively occurred towards males of the 'right' species and never against heterospecific males (Fisher's exact test, intra- versus interspecific courtships:  $p=0.0005$ ).

If a female was unwilling to mate, she either remained completely calm (more rarely), or she fluttered with her wings, but remained in place. Alternatively, the female flew off. Even with intraspecific interactions, the majority of courtships did not result in a mating (*L. reali*: 88% of courtships; *L. sinapis*: 73%). Occasionally (Table 6) males tried to mate with a female even though she had not signalled receptiveness. Such attempts were never successful.

We observed 12 intraspecific copulations (Fisher's exact test, intra- versus interspecific courtships:  $p=0.0015$ ). In the four cases where no copulation occurred, despite the female's willingness, the male was always hindered by some obstacles (such as plant twigs or leaves) to achieve the proper mating position. Copulations lasted  $47.1 \pm 32.7$  min ( $n=7$ ) in *L. reali* and  $63.2 \pm 32.2$  min ( $n=5$ ) in *L. sinapis* with no significant difference between the two species (*t*-test:  $t=0.387$ ,  $p>0.7$ ). Though variance was very large, courtships leading to successful matings were longer (median 20 s,

n=8) than unsuccessful intraspecific courtship sequences (median 6 s, n=63; Kolmogorov-Smirnov test:  $p < 0.025$ ).

Not only young females were successfully courted. Two females aged four or five days, respectively, but still virgin, were accepted by courting males. With 36 and 48 min, respectively, these two matings fell well within the variation observed with younger females. The 5 d-old *L. reali* female subsequently laid 25 fertile eggs, whereas the 4 d-old *L. sinapis* female died accidentally without having laid eggs in captivity.

## Discussion

**Ethological reproductive isolation.** Our experiments in a large flight cage revealed that the females of *L. reali* and *L. sinapis* clearly distinguish between these two species. Males courted heterospecific females with the same intensity as conspecifics (measured by courtship duration), whereas females never signalled receptiveness towards heterospecific males (evidenced by forward bending of the abdomen). Species discrimination was less straightforward in males. While *L. reali* males very rarely courted *L. sinapis* females, *L. sinapis* males appeared not to discriminate against *L. reali* females when courting. Neither in the laboratory nor in the field (A. Freese, unpublished observations, identity of mates confirmed subsequently by dissection after their death) did we observe interspecific matings. These observations provide strong evidence for a precopulatory mate choice particularly (but not only) through the female sex, in contrast to predictions derived from the lock-and-key hypothesis which would suggest that species discrimination occurs only at insertion of the male's genitalia into the female's copulatory opening.

Due to the limited simultaneous availability of adult butterflies in sufficiently large numbers, our mating experiments could not be fully standardized. It was impossible to stock the cage for each observational sequence with the same number of individuals of all sexes and species. For example, *L. sinapis* females were in rather short supply which might have influenced our results. Moreover, the number of observational replicates was low. Some butterflies were present in the flight cage during more than one observation session, and some degree of pseudoreplication also occurred since the same individual butterflies happened to interact with other individuals in the cage more than once within one observational session. From all these reasons, the statistical results derived from our observational data should not be overemphasized, and further tests under improved conditions might produce slightly different results. Most animals exhibited behaviours such as food searching, courtship and mating in much the same way as in nature. The frequent rejection of courting males by virgin females was not a laboratory artefact, but also often occurred in the field (A. Freese, unpublished observations). This contrasts with Wiklund's notion (1977a) according to which only mated females would exhibit avoidance behaviour against courting males.

Our experiments with northern Bavarian stock support the concept of *L. reali* and *L. sinapis* being distinct biospecies, whose reproductive isolation is maintained by precopulatory ethological isolation mediated through (mostly female) mate choice. Initial evidence for such ethological isolation was reported by Lorkovic (1993) from



Croatia. Thus, species recognition and reproductive isolation are not just regionally limited phenomena, as it occasionally occurs in ring species or 'super-species' (Barton & Hewitt 1985, Harrison 1990). From his experiments on reproductive isolation between '*L. sinapis*' (in retrospect it is unclear whether he experimented with true *L. sinapis* or *L. reali*) and *L. morsei* Fent., Lorkovic (1950) also concluded that the female sex controls species-specific mating, while males attempt interspecific hybrid pairings.

The role of sexual selection and female choice in speciation has been emphasized in mathematical models (e.g. Fisher 1930; Lande 1981; Kirkpatrick 1982) and has gained increasing empirical support (Panhuis *et al.* 2001). Sexually selected signals are important in speciation processes (Darwin 1871; Thornhill & Alcock 1983; West-Eberhard 1983). For example, Wiernasz (1989) and Wiernasz & Kingsolver (1992) showed that in the two closely related and morphologically similar species *Pieris occidentalis* and *P. protodice* no hybrids occur in nature, although postcopulatory isolation barriers do not exist. In this case the degree of melanization of the fore wings serves as recognition signal for the female during mate choice.

The nature of signals responsible for precopulatory species discrimination between *L. sinapis* and *L. reali* remains to be uncovered. Wing melanization is unlikely to play an important role since it strongly varies in both species between generations (Mazel 2000, 2001a). Only extreme phenotypes look so different to the human observer as to allow for species distinction. Lorković (1930) assumed that species-specific pheromones mediate recognition between *Leptidea morsei* and '*L. sinapis sensu lato*', and this also seems the most likely explanation in the sibling species pair *L. sinapis* / *L. reali*.

Androconia are well known from many Pieridae (Halfter *et al.* 1990), and their pheromones are important in sexual interactions (Omura *et al.* 2000). Close inspection under a stereomicroscope (50-fold magnification) of both *Leptidea* species did not reveal any morphologically distinct androconia on the wings (A. Freese, unpublished observations). This does, however, not imply that there are no glands that could dissipate a male sex pheromone. Clearly, the nature and source of this isolating signal deserves further research.

Hybridization experiments by Lorkovic (1950) between '*L. sinapis sensu lato*' and *L. morsei* revealed that enforced copulations were only possible after specialized scales around the genital opening of the female had been removed experimentally. Hence in *Leptidea* another factor is involved in reproductive isolation which again has nothing to do with the size and shape of the sclerotized genitalia apparatus. Hybrids achieved from these experiments were viable, but completely sterile. Whether this mode of reproductive isolation is also acting between *L. sinapis* and *L. reali* has not been studied thus far.

**Ecological differentiation.** Our experiments revealed that apart from ethological isolation between *L. reali* and *L. sinapis* ecological differences also exist. Females ranked the four tested hostplant species differentially, with *L. reali* preferentially ovipositing on *Lathyrus pratensis*, while this plant ranked second-lowest in *L. sinapis* (which preferred *Lotus corniculatus*). These experimental findings are exactly in line with the hypothesis about hostplant preferences advanced by Lorković (1993) and

Kristal & Nässig (1996). However, in both species some individuals showed oviposition preferences of the 'opposite' species, suggesting that this differentiation is not yet fixed in either of the species and may also vary regionally. For example, in the vicinity of Bayreuth egg-laying of females (identity subsequently confirmed by dissection) occurred on *L. pratensis* as well as *L. corniculatus* (A. Freese, unpublished observations). Hence, without controlling for individual preferences and the local availability of oviposition substrates, chance field observations could well be misleading for inferences about hostplant preference hierarchies (Tabashnik *et al.* 1981, Rausher & Papaj 1983, Thompson & Pellmyr 1991). *M. sativa* was for both *Leptidea* species the most unattractive oviposition substrate, which is in accordance with the rarity of records of this plant in the literature. *L. corniculatus* and *L. pratensis* are cited most often, followed by *V. cracca*, and *Medicago* is mentioned least often (e.g. Wiklund 1977b; Henriksen & Kreutzer 1982; Warren 1984, Thomas & Lewington 1991; Bink 1992; Ebert & Rennwald 1993).

Minor differences between important life-history parameters of both species also emerged in the rearing experiments under fully standardized environmental conditions. *L. reali* grew slightly larger (in particular so in the male sex) and took about 1d longer to develop, whereas *L. sinapis* had higher growth rates during the final larval instar. This might indicate that selection has favoured life-history evolution towards larger body size in *L. reali* as opposed to more rapid development in *L. sinapis*. However, variation was pronounced and it remains to be tested whether these subtle differences would be important under more variable natural growth conditions or would recur with animals from geographically distant populations.

Under the rearing regime (high constant temperature, long photoperiod) no individual entered diapause and all passed through four larval instars only (cf. Warren 1984). Development across five larval instars, as occasionally reported in older sources as being characteristic for first generation larvae (Emmet & Heath 1990), never occurred. We can at present not ascertain whether these *Leptidea* species never pass through a five-instar pathway. In other butterflies, instar number is indeed a more plastic trait that varies between populations and may also be associated with diapause or subitaneous development (Fischer & Fiedler 2002).

Both *Leptidea* species exhibited distinct protandry, with development time being about 2d shorter in males (cf. Wiklund & Solbreck 1982). The extent of protandry did not differ between species. Protandry was realized by a combination of higher growth rates and smaller body size in males, while the females took more time (mainly in the final larval and pupal stage) and grew about 10% larger. Larger body size in females probably reflects selective advantages such as increased fecundity (Wiklund & Karlsson 1988, Wickman & Karlsson 1989, Honek 1993). This should be particularly relevant in egg-limited insects like *Leptidea* species with a mean lifetime fecundity of but 30–40 eggs.

Protandry should be selected for if females mate only once and virgin females quickly become rare during population emergence (Wiklund 1977a; Wiklund & Solbreck 1982; Zonneveld & Metz 1991). Under such conditions, males may be forced to accept severe trade-offs between body size and speed of development (Fischer & Fiedler 2001). Larval food plant affected most life-history traits in no-choice experiments, but spe-



cies-specific effects (i.e. species  $\times$  food interactions) were seen only with growth rates. *L. reali* achieved relatively low growth rates when fed *V. cracca* and *L. pratensis*. Overall, however, the different plant species offered in our experiments had largely similar effects on performance and fitness of *L. reali* and *L. sinapis*. *L. corniculatus* turned out to be the most favourable plant with regard to body size and duration of development. *L. pratensis* and *V. cracca* were almost equally suitable for both species, while *M. sativa* was overall the least favourable plant. Mortality (*L. reali*:  $66.2 \pm 16.6\%$ ; *L. sinapis*:  $49.9 \pm 30.0\%$ ) of larvae was also highest when fed *M. sativa*. Thus, the hierarchy of food plants according to larval performance was for both *Leptidea* species *L. corniculatus*  $>$  *L. pratensis* = *V. cracca*  $>$  *M. sativa*.

In choice situations larvae of both *Leptidea* species preferred plants almost exactly as would be expected according to the performance hierarchy. Discrimination against *M. sativa* was very strong, and also both species clearly favoured *L. corniculatus*. In addition, feeding experience had a strong influence on feeding choices. Remarkably, earlier feeding on *L. pratensis* did not increase the likelihood of accepting that same plant later in a choice situation. Even in *L. reali* (where females prefer *L. pratensis* for oviposition), more *Lathyrus*-fed larvae switched to *Lotus* than vice versa.

Although in Lepidoptera with relatively sedentary larvae the female's choice of a hostplant for oviposition has usually the highest impact on larval survival, the ability to make a choice also may have selective advantages for the caterpillars. For example, when caterpillars fall off their hostplant after an attack, or if a plant individual does not provide sufficient resources, larvae must be able to find and select a proper new hostplant (Bernays & Chapman 1994).

While the caterpillars' choices perfectly matched their performance, egg-laying decisions and offspring performance showed a discrepancy in *L. reali*, where a plant species offering less than optimal performance (i.e. *Lathyrus pratensis*) was preferred for oviposition. Theoretically one would expect female preference and offspring performance to be tightly correlated to maximise fitness (Thompson & Pellmyr 1991; Gratton & Welter 1998). However, apart from phytochemical and nutritional differences between hostplant species, factors such as the incidence of predators, parasitoids or competitors may cause discrepancies between preference and performance (Thompson 1988a; Thompson & Pellmyr 1991). Thus, the validity of the performance hierarchies, as extracted from our experimental study, needs to be assessed under field conditions. Yet there is thus far no reason to assume that levels of predation, parasitism, or competition should differ between, for example, *L. corniculatus* and *L. pratensis* in the case of the two *Leptidea* species.

Oviposition preferences of butterflies are often heritable and vary within and across populations (Thompson 1988b; Singer *et al.* 1988; Nylin & Janz 1996). Although we did not test for heritability of preferences, individual variation was pronounced in both *Leptidea* species. Any reduction in gene flow between both forms (with genetically different preferences) should, on the long run, improve the preference-performance correlation within each group (Via 1986). The discrepancy between preference and performance in *L. reali* could then be explained by the hypothesis that the time passed



since the split of the two taxa has not yet been sufficient for a clear preference-performance correlation to evolve (Thompson 1988a).

In herbivorous insects, heritable differences in hostplant preferences may be the driving force towards speciation, even in sympatry, provided that genetical preference-performance correlations or assortative hostplant related matings occur (e.g. Felsenstein 1981; Via 1986, Singer *et al.* 1988). In *L. sinapis* and *L. reali*, with their strong overlap with regard to larval hostplants and the only incipient segregation with regard to preference hierarchies, it seems very unlikely that these subtle differences have caused or even facilitated speciation. Rather, it is likely to assume given the results presented here that incipient speciation was mediated by sexual selection and female mate choice, with the weak ecological segregation evolving as a chance by-product (possibly via genetic drift: Schluter 2001).

**P r o s p e c t s .** The results presented here demonstrate that *L. sinapis* and *L. reali* are in all likelihood two different biospecies separated by ethological reproductive isolation barriers. The two species are only weakly differentiated in ecological terms, and speciation may not yet have reached the level of complete interruption of gene flow. For example, the occasional occurrence of individuals with 'odd' genitalia measures in the offspring of females of both species (A. Freese and K. Fiedler, unpublished results) might indicate that limited introgression still takes place. To test this possibility rigorously, measures of gene flow by means of allozyme electrophoresis or DNA techniques will be required (Geiger 1988; Pollock *et al.* 1998).

The notion that '*L. sinapis* sensu lato' in fact comprises a sibling species pair also raises the question as to whether earlier ecological studies on the species complex remain valid (e.g. Wiklund 1977a, 1977b; Wiklund & Solbreck 1982; Warren 1985; Warren *et al.* 1986). In retrospect, it will be difficult to unequivocally determine with which of the two species these studies were done (unless voucher specimens were still retained). Distributional areas of both species overlap widely in Europe, and even syntopic occurrences are known. Our investigations demonstrate the very strong similarities between both *Leptidea* species in terms of ecology and life-history. Also with regard to nature conservation issues, problems recognized for '*L. sinapis* sensu lato' (Dennis 1977; Warren 1985; Warren *et al.* 1986; SBN 1987; Ebert & Rennwald 1993) are most likely relevant for both of its component species. For example, all hostplant species are restricted to rather early successional stages of vegetation, fecundity is equally low, and there is much overlap in body size, emergence times or longevity. Thus, threats to the existence of one species will also affect the other, and recovery from population reductions should also not differ. Nevertheless, the case of the two Wood Whites is again a reminder that it is not only most worthwhile to examine putatively 'common' and 'well-known' species more thoroughly, but also to fully document results and retain voucher specimens for subsequent validation.

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