

Reproductive isolation between populations from Northern and Central Europe of the leaf beetle *Chrysomela lapponica* L.

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Summary. Allopatric populations of the leaf beetle *Chrysomela lapponica* are known to feed upon either willow (Salicaceae) or birch (Betulaceae). This study aimed to elucidate the differentiation process of these allopatric populations. We investigated whether these allopatric populations specialized on different host plants are still able to produce fertile offspring when interbreeding. Individuals from a population in Finland (willow specialists) and one in the Czech Republic (birch specialists) were crossed in laboratory. Hybrid formation succeeded only between females from the Czech, birch specialized population and males from the Finnish, willow specialized population, while no eggs were produced by females of the willow specialists having mated males of the birch specialists. Behavioral, morphological, physiological, and chemical features of the F₁ hybrids were studied. The chemical composition of larval defensive secretion and feeding preferences of the resulting F₁ hybrids mainly showed similarities with the paternal phenotype, while the area of black coloring on the offspring's elytra was intermediate between those of the parental elytra. F₁ hybrids did not accept the host plant (birch) of their mothers for feeding and only survived on willow. Thus, since mothers only lay eggs on birch, we found evidence for a postzygotic isolation mechanism between the individuals of the two investigated populations: when having been mated with willow specialized Finnish males, the birch specialized Czech mothers place the hybrid eggs on a plant species (birch), on which the hatching larvae cannot survive.

Key words. *Chrysomela lapponica* – speciation – hybridization – Salicaceae – Betulaceae – host plant specialization – host plant shift – post zygotic isolation mechanism – heterosis

Introduction

The vast majority of herbivorous insects shows a high specialization for their host plants. Most species feed on one or few host plant species which are usually closely related (Bernays & Chapman 1994; Farrell & Mitter 1993; Jermy

1984). Since decades there has been an interest in analyzing the factors that have driven host plant shifts in phytophagous insects (Becerra 1997; Ehrlich & Raven 1964; Futuyma & Keese 1992; Futuyma & McCafferty 1990; Gross *et al.* 2004b; Gross *et al.* 2004a). Several hypotheses have been proposed with respect to the evolution of host plant use in herbivorous insects. The first hypothesis states that host plant shifts of phytophagous insects between distantly related plant families might become successful due to phytochemical similarities (Becerra 1997; Ehrlich & Raven 1964; Futuyma & McCafferty 1990; Köpf *et al.* 1998). Secondly, a so called parallel cladogenesis may have occurred. This has been found in some herbivore groups (Farrell & Mitter 1990; Mitter *et al.* 1991). A third hypothesis suggests that within the same geographical area of the ancestral host plant switches to a new plant species may evolve, if these plant species are sympatrically distributed (Bernays & Chapman 1994; Köpf *et al.* 1998; Pasteels & Rowell-Rahier 1991).

Chrysomelid beetles represent an excellent model to investigate the ability of specialized phytophagous insects shifting among hosts and thereafter forming new species. Indeed, most of the species are highly specialized in their feeding habitats. Their whole life cycle occurs on the same plant, and a single host plant may influence a large number of performance parameters (Gross *et al.* 2004a; Jolivet & Hawkeswood 1995; Jolivet & Verma 2002). This pattern of narrow association might be highly conservative in the evolutionary process. Studies on the genera *Phyllobrotica* and *Ophraella* showed host plant switches only within the same plant genus (Farrell & Mitter 1990; Futuyma & McCafferty 1990; Mitter *et al.* 1991). Nevertheless, in some genera like *Oreina*, *Gonioctena*, *Lochmaea*, *Chrysomela*, and *Phratora*, radical shifts between distantly related plants have occurred (Dobler *et al.* 1996; Gross 2001; Köpf *et al.* 1998; Mardulyn *et al.* 1997; Mikheev 1998; Termonia *et al.* 2001). Here, the occurrence of host shifts may be explained by chemical similarities of the plants or by their sympatric growing.

Colonization of a new host plant may lead to a change of host plant preferences, subsequently to a loss of use of the ancestral plant, followed by reproductive isolation of the individuals on the newly pioneered plant, thereby forming a new host race (Gross *et al.* 2004a; Peterson & Denno 1998).

New host race formation can take place even in sympatry because a newly exploited host plant with possibly different phenology or morphology may lead to a reduced gene flow between the two host races and finally result in reproductive isolation (Feder *et al.* 1994; Linn *et al.* 2004; Nokkala & Nokkala 1994). Few examples of host race formations are known in chrysomelids like in the *Galerucella nymphaeae* species complex (Hippa & Koponen 1986; Nokkala & Nokkala 1994, 1998; Pappers *et al.* 2002a, 2002b) or in *Lochmaea caprea* (Kreslavskiy & Mikheyev 1994). However, in these examples hybrid formations are still possible between the two host races. Moreover, an interspecific hybridization is known from *Diabrotica virgifera* and *D. longicornis* in laboratory (Krysan & Guss 1978), and hybrids between the leaf beetle species *Chrysochus auratus* and *C. cobaltinus* were found in nature (Peterson *et al.* 2001).

The leaf beetle species *Chrysomela lapponica* (L.) belongs to the *C. interrupta*-group, which is distributed mainly in the Nearctic. Most species of this taxon feed upon their ancestral host plants belonging to Salicaceae, while a few species (e.g. *C. interrupta*, *C. mainensis*) shifted to Betulaceae (Brown 1956; Gross 1997; Gross *et al.* 2004b; Gross & Hilker 1995; Termonia *et al.* 2001; Termonia & Pasteels 1999). *C. lapponica* is the only Palaearctic species belonging to the *C. interrupta*-group. It presents a highly interesting model to study the evolution of host plant specialization because distinct allopatric populations exist which show specialization on either birch species (*Betula pubescens*, *B. pendula*; Betulaceae) or willow species, in particular *Salix borealis* (Salicaceae), even though both birches and willows occur side by side in the habitats. Up to now, populations from the Czech Republic, Germany, and Poland are known to feed on birch (Gross 1997; Gross *et al.* 2004a; Hilker & Schulz 1994), whereas populations from Finland, France, Norway, and Russia are found to feed on willow (Gross 2001; Gross *et al.* 2004b; Hilker & Schulz 1994; Machkour M'Rabet 1996; Zvereva *et al.* 1995).

Earlier investigations showed a high adaptation of the two allospecies to their host plant and their chemistry (Gross *et al.* 2004c; Gross & Fatouros 2006; Gross & Hilker 1995; Hilker & Schulz 1994; Schulz *et al.* 1997). All larvae of the genus *Chrysomela* discharge defensive secretions from nine pairs of dorsal glands, when disturbed. Particularly, a change in the composition of the larval secretion is associated with the specialization of *C. lapponica* on either birches or willows. These exocrine secretions are well known to act as repellents towards predators (Blum *et al.* 1972; Gross 2001; Hilker & Schulz 1994; Pasteels *et al.* 1988) or entomopathogenic fungi (Gross *et al.* 1998; Gross *et al.* 2002). Most *Chrysomela* species rely on the ancestral pathway to biosynthesize their larval secretion, i.e. they sequester the plant toxin salicin, hydrolyze and oxidize it to release salicylaldehyde as major component (Pasteels *et al.* 1983). In contrast, the larvae of the *C. interrupta*-group have developed a new biosynthetic pathway, the esterification of *de novo*-synthesized butyric acids by alcohols retrieved from the plant (Schulz *et al.* 1997).

In the present study, we aimed to elucidate the compatibility of allopatric populations of *C. lapponica* with different host plant preferences. We addressed the question

whether individuals of two allopatric populations of *C. lapponica*, one distributed in Northern Europe (Finland) feeding on willow and one from Central Europe (Czech Republic) feeding on birch, are able to hybridize at laboratory conditions. If successful interbreeding would be possible we addressed the question how do the F₁ hybrids perform and which host plant do they prefer.

After successful interbreeding, we studied the viability of the eggs and feeding preferences of the surviving larvae and adults. Moreover, we analyzed the main components of the defensive secretion of the hybrid larvae by GC-MS and the elytral coloration patterns of the adults. Because a recent study showed significant longer developmental times of birch feeding larvae of *C. lapponica* compared to larvae that developed on willow (Gross *et al.* 2004a), we also examined the developmental time of the hybrids that were only able to feed upon willow.

Material and methods

Insects and plants

Adults of *C. lapponica* of a birch specialized population from a peat bog close to Kladská (Czech Republic, 50°00'N 12°40'E) as well as of a willow specialized population from Finnish Lapland in the surroundings of the Kevo Subarctic Research Station (69°45'N 27°01'E) were collected at the end of the vegetation period and transferred to a climate chamber, where they overwintered in plastic boxes (15 × 15 × 6 cm) filled with humid moss at 6 °C and 24 h darkness. Seven months later, the overwintering was terminated by changing the conditions to 15 °C, 70 % RH and L:D 16:8 h. This was conducted simultaneously for both populations. Thus, we were able to synchronize the receptivity and willingness to mate in both populations. Because of the low number of surviving individuals of the birch-feeding population, additionally adult beetles that overwintered in their natural habitat in Kladská were collected from the leaf litter layer, directly at the beginning of the growing period. They were transferred to laboratory and kept under the same conditions. These beetles were used as control group, only. Males and females of each population were kept first separately and fed with leaves of *Betula pubescens* (Czech population) or *Salix fragilis* (Finnish population) obtained from trees in the botanical garden of the "Freie Universität Berlin", Germany. We used *Salix fragilis* as food plant for the Finnish population, because their natural host plant *S. borealis* (only distributed in Northern Fennoscandia) was not available for the experiments. But both *S. borealis* and *S. fragilis* are similar in their chemical constitution, mainly differing in their content of phenol glucosides (Julkunen-Tiitto 1989). After a feeding period of 3 weeks females and males were put together in four different pairings, to conduct the crossing experiments (Table 1).

Cross breeding experiments

Crossing experiments between males of the willow specialized Finnish population and females of the birch specialized Czech population (N=21) and vice versa (N=7) were conducted. For control, again crossings within populations were conducted (Finnish population: N=19 pairs; Czech population: N=10 pairs). The beetles were kept pairwise at 15 °C, 70 % RH and L:D 16:8 h for four weeks. For the within-population crossings of the Czech control groups, all pairs were kept together in one box, while for all other crossings the pairs were kept separately. Individuals of all crossings were supplied with twigs of host plants, both *Salix fragilis* and *Betula pubescens*, placed in water-filled vials into the boxes. Every second day the twigs were replaced by fresh ones and newly laid egg batches were removed.

Table 1. Overview of crossings with individuals of *C. lapponica*: CR from the birch-specialized population of Czech Republic (within population crossing CR × CR; control group), FI from the willow-specialized population of Finland (within population crossing FI × FI, control group) and their interbreeding crosses (CR × FI; FI × CR, test groups). Percent reproduction refers to the number of pairs that produced offspring. Total number off eggs laid is given.

Origin (female × male)	N pairs	% Reproductive (N pairs)	N eggs
CR × FI	21	23.8 (5)	244
FI × CR	7	0	0
CR × CR*	10	n. p.	344
FI × FI	19	21.1 (4)	216

* Adults overwintered in the field; n.p. = not provable

Quantification of larval secretion volume

Larval secretion was collected from 3rd instar larvae of the F₁ hybrids of *C. lapponica* obtained from crossing of females from the Czech population with males from the Finnish population. F₁ larvae (N=5) were fed with leaves of *Salix fragilis*. The larvae were stimulated with forceps on the back until secretions were released from the glandular reservoirs. The liquid secretion emitted by all 18 reservoirs of each larva was sucked into a calibrated glass capillary (intraMark 5 µl, Blaubrand, Germany), which was subsequently melted at both ends. The volume was calculated from the measured length between the menisci of the secretion inside the capillary and the capillary diameter.

GC-MS analysis of larval secretion

The chemical composition of the larval secretions from the two parental *C. lapponica* populations (Finland, Czech Republic) feeding either upon *Betula pubescens* or *Salix borealis* has already been analyzed and presented in an earlier publication (Gross *et al.* 2002). In this study, we analyzed the composition of the larval secretion of the F₁ hybrid generation obtained from crossing of females from the Czech population with males from the Finnish population by coupled gas chromatography-mass spectrometry under the same conditions as in the previous study and compared it qualitatively and quantitatively with the chemical composition of larval secretions of the parental generations on their respective host plants. F₁ larvae were fed with *S. fragilis* and secretion was collected in capillaries. Accordingly, secretion was dissolved in 20 µl of dichloromethane containing dodecane as internal standard (IS; 0.1 µg/µl; Aldrich, Germany), and 1 µl was injected splitless into a gas chromatograph (Fisons GC 8060, Thermoquest, Germany, injector temperature 240 °C), equipped with a 30 m × 0.32 mm × 0.25 µm db-5 column (Fisons). The temperature program started at 40 °C was held for 4 min and raised with 10 °C/min to 280 °C. Helium (Air Liquide, Germany) was used as carrier gas (inlet pressure 10 kPa). The gas chromatograph was coupled to a quadrupole mass spectrometer (Fisons MD 800; Thermoquest). EI mass spectra were recorded at 70 eV. The components were identified as described by Gross *et al.* (2002). Selected components were quantified as following: Comparing the peak area of the IS to the area of the respective component, a ratio depending on concentration was calculated. The concentrations of the specific components (µg/µl) was calculated using following formula: $A \times B \times C/D \times 1000$ (A: ratio peak area of IS/peak area of component; B: slope of calibration curve of synthetic reference substance [ng/µl]; C: secretion volume + 20 µl DCM [µl]; D: secretion volume [µl]). Quantitative data were obtained by analyses of the secretions from five hybrid larvae.

Coloration of the elytra

In order to quantify the pattern and proportion of black and red coloring, the left and right elytra of 20 adult F₁ hybrids were examined. The areas of the red and black marks were determined by

covering the elytra with a transparent plastic foil on which a grid (0.1 × 0.1 mm) was imprinted. A binocular eyepiece was used for measuring the areas. The degree of melanization was calculated as percentage area with black coloring of the elytral surface and compared statistically with data from the two parental allospecies (Gross *et al.* 2004c).

Fecundity and egg viability

Egg batches of all parent combinations (Table 1) were transferred to Petri dishes (diameter 9 cm). Eggs were counted and the number of hatching larvae was recorded. The fecundity of females was determined by counting the number of eggs per female. Since within-population crossings of the pairs from the Czech population were not held separately (see above), only the number of eggs per clutch could be determined.

Survival, developmental time and body weight

Larvae were kept from hatching until pupation in groups of about 30 individuals (1st and 2nd instars) or 10 individuals (3rd instar) in Petri dishes (diameter 9 cm) in a climate chamber at 15 °C, 70 % RH, and L:D 16:8 h on leaves of *S. fragilis* or *B. pubescens*. The conditions were the same as for the parental generations, tested in an earlier study (Gross *et al.* 2004a). The leaves were removed and replaced by fresh ones every second day, over the whole experimental time. Every day the numbers of alive and dead individuals and their ontogenetic stages were recorded. The total developmental time was calculated from the hatching day of the neonate larvae until they have reached the adult stage, because the duration of the egg stage cannot be influenced by the host plant. Body weights of newly emerged adults of the F₁ hybrids fed with *S. fragilis* were measured and compared with body weights from the birch-specialized and willow-specialized populations, reared under the same conditions on their natural host plants or on *S. fragilis* (Gross *et al.* 2004a).

Feeding preferences

The feeding preferences of both larvae and adults of the hybrids were measured by dual choice feeding bioassays conducted at 15 °C, L:D 16:8 h. An adult or a 3rd instar larva (N=14) was placed into a Petri dish (diameter 9 cm) lined with moistened filter paper (400 µl aqua dest.) and offered a choice between two fixed leaf discs (diameter 12.5 mm) of both *S. fragilis* and *B. pubescens* for 22 h. The amount of leaf material eaten was recorded by scanning leaf discs and calculating the remaining leaf area by the computer program Bioproject 3.0.

Statistical analysis

Differences between the concentrations of the quantified compounds of larval secretions as well as the volumes of larval secretion, elytral coloration measurements, body weight, fecundity

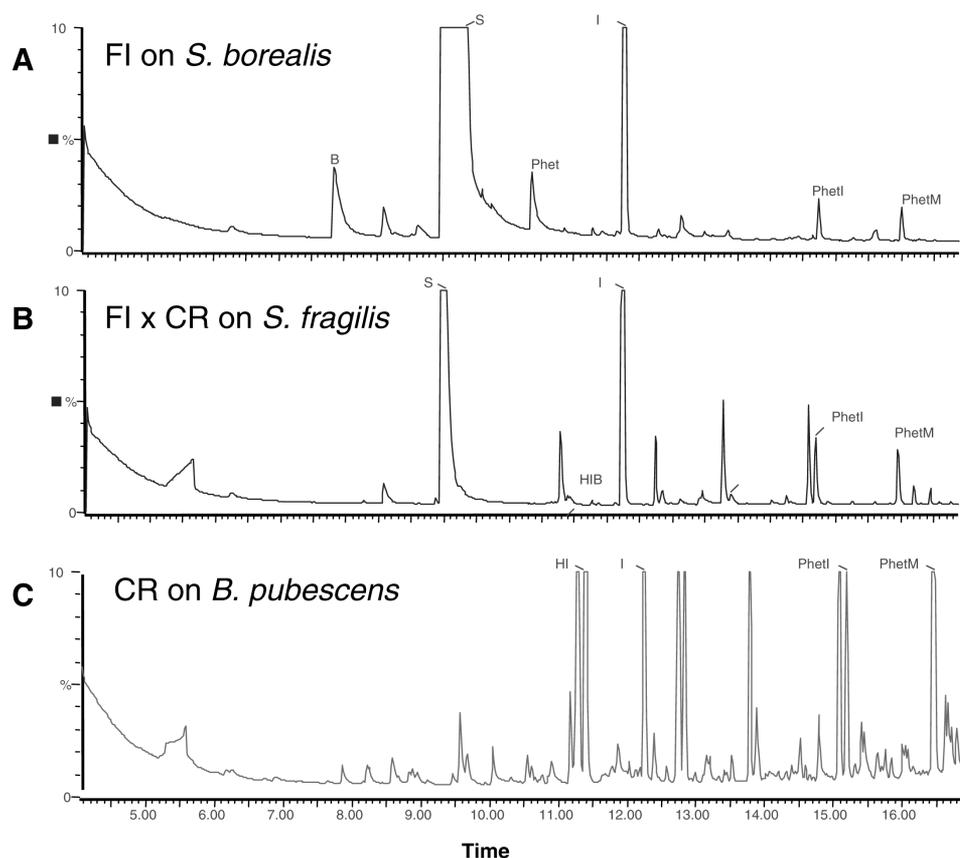


Fig 1. Chromatograms of larval exocrine secretions of both parental populations and resulting hybrids. A: Population from Finland (FI) fed with *Salix borealis*; B: Hybrids between the two populations (FI \times CR) fed with *Salix fragilis*; C: Population from the Czech Republic (CR) fed with *Betula pubescens*. Abbreviations: B: Benzaldehyde; HI: Hexyl isobutyrate; HIB: (*Z*)-3-Hexenyl isobutyrate; I: Internal standard; Phet: Phenyl ethanol; PhetI: 2-Phenylethyl isobutyrate; PhetM: 2-Phenylethyl 2-methylbutyrate; S: Salicylaldehyde

and developmental times were statistically compared between the individuals of different populations and the F_1 hybrids by the Mann-Whitney U-test (Sachs 1992). Multiple comparisons of data were confined to Bonferroni-correction. The egg viability and mortality rates of beetles reared on different host plants were analyzed by χ^2 -tests. The Wilcoxon signed rank test for paired samples was used for analysis of feeding preferences of the hybrid larvae and adults. All statistical analyses were conducted using the Statistica 5.5 software (StatSoft 1999).

Results

Crossbreeding

From 21 interbreeding crosses between females of the birch specialized population from the Czech Republic and males of the willow specialized population from Finland (CR \times FI), five pairs (23.8 %) produced 244 eggs, in total. None of the seven pairs of the reciprocal interbreeding crosses produced offspring. Within the control group of the birch specialized population (CR \times CR) 344 eggs were laid, whereas 21.1 % of the pairs of the willow specialized population (FI \times FI) produced 216 eggs, in total (Table 1).

Chemical traits of the larval secretion

Composition

GC-MS-analyses of larval secretions of populations of *C. lapponica* feeding either upon birch or willow have already been published (Gross & Hilker 1995; Gross *et al.* 2002; Hilker & Schulz 1994). Also the larval secretion of the F_1 hybrids having been fed with *S. fragilis* revealed the presence of known components. But remarkable differences were detected (Fig. 1, Table 2a). Like the secretion of willow feeding larvae of the paternal population from Finland, the hybrid larval secretion contained salicylaldehyde as major component, but neither benzaldehyde nor 2-phenylethanol. The secretion of the hybrid larvae contained numerous isobutyric and 2-methyl butyric esters. Four ester components were quantified (Table 2a). In contrast, the larval secretion of the maternal population from the Czech Republic contained significantly higher amounts of these substances. Whereas (*Z*)-3-hexenyl isobutyrate could not be detected in the secretion of the paternal Finnish larvae feeding upon *S. borealis*, the hybrid secretion did

Table 2. Statistical comparison of the main traits between different life stages of two allopatric populations of *C. lapponica* from the birch-specialized Czech population (CR), the willow-specialized Finnish population (FI) kept on their natural host plant (mainly literature data), and their hybrids (CR (female) × FI (male)). Mean values and standard deviations are given. **a)** n=5. **b)** n=5. Mann-Whitney-U test: (p<0.05) **c)** n=20 **d)** CR n=101; FI n=179; CR × FI n=73. χ^2 -test: p<0.01 **e)** FI n=4, CR × FI n=5 **f)** n clutches: CR n=11, FI n=6, CR × FI n=6.

Origin Fed with	Czech Republic (CR) <i>Betula pubescens</i>	Finland (FI) <i>Salix borealis</i>	F ₁ Hybrid CR × FI <i>Salix fragilis</i>
CHEMICAL TRAITS OF LARVAL SECRETIONS			
a) Major compounds¹			
Benzaldehyde	0	0.20 ± 0.21 ^a	0
Salicylaldehyde	0	58.36 ± 31.08 ^a	29.18 8.96 ^a
2-Phenylethanol	0	0.11 ± 0.11 ^a	0
Hexyl isobutyrate	0.76 ± 0.12 ^a	0.01 ± 0.01 ^b	0.02 ± 0.03 ^b
(Z)-3-Hexenyl isobutyrate	0.88 ± 0.03 ^a	0	0.19 ± 0.13 ^b
2-Phenylethyl isobutyrate	0.84 ± 0.11 ^a	0.05 ± 0.05 ^b	0.20 ± 0.11 ^b
2-Phenylethyl 2-methylbutyrate	1.01 ± 0.14 ^a	0.02 ± 0.02 ^b	0.15 ± 0.04 ^c
b) Volume²	0.20 ± 0.08 ^a	0.48 ± 0.25 ^a	0.22 ± 0.06 ^a
MORPHOLOGICAL TRAITS OF ADULTS			
c) Proportion black coloring of elytra³	51 ± 7.3 ^a	68 ± 9.2 ^b	57 ± 5.9 ^c
PERFORMANCE TRAITS			
d) Egg viability⁴	78.8 ^a	85.1 ^a	32.8 ^b
e) Fecundity⁵	45.7 ± 16.41 ^a	54.0 ± 30.77a	48.8 ± 51.20 ^a
f) Eggs/clutch	31.3 ± 8.16a*	36.0 ± 10.37a	34.8 ± 2.14 ^a

^{a,b,c} Different letters indicate significant differences between columns. The different statistical methods are described in the text. ¹ Concentration (µg/µl). Data in italics from Gross et al. 2002; ² volume [µl]. Data in italics from Gross et al. 2004a; ³ black coloring [%]. Data in italics from Gross 2001; ⁴ n hatched larvae. Data in italics from Gross et al. 2004a; ⁵ n eggs per female; Data in italics from Gross et al. 2004a. All other data this paper.

contain this compound. The ester 2-phenylethyl 2-methylbutyrate was found in significantly higher amounts in the hybrid secretion compared to the paternal Finnish secretion (p<0.05, Mann-Whitney U-test).

Volume of larval secretion

No differences were found between the volume of larval secretion emitted by the willow feeding F₁ hybrids (0.2 ± 0.06 µl) and their parental populations, when they were fed with their natural host plants *S. borealis* (FI) or *B. pubescens* (CR), respectively (Table 2b, p>0.05, Mann-Whitney U-test). But the secretion volume of F₁ hybrid larvae was significantly higher than of larvae from the maternal Czech population, when they were fed with *S. fragilis* (Gross et al. 2004a).

Morphological traits

Coloration of the elytra

The black marks take about 57 ± 5.9 % (N=20) of the brightly red elytra area of the adult F₁ hybrids (Table 2c). While the percentage of the black color was 51 ± 7.3 % (N=20) in individuals from the maternal Czech population, the black elytral proportion of the paternal Finnish morphs was in average 69 ± 9.2 % (N=20) (Gross et al. 2004c). A statistical comparison of the black and red coloring

proportions of the hybrids to those of the parental populations revealed that the hybrid elytra were significantly darker than those of the beetles from the maternal population (Czech Republic), but significantly lighter than of those of the paternal one from Finland (Mann-Whitney U-test, p<0.001).

Performance traits

Fecundity and egg viability

Crossings between females of *C. lapponica* from the birch specialized Czech population (CR) and males from the willow specialized Finnish (FI) population produced offspring which had a low egg viability (32.8 %; i.e. 80 larvae out of 244 eggs) (Table 2d). The egg viability of the birch specialized control group (CR × CR) was much higher (78.8 %) than that of the hybrids CR × FI. No larvae hatched from the eggs of the willow specialized control group (FI × FI) because of contamination and destruction by molts. Instead data of a previous investigation (Gross et al. 2004a) were taken into statistical comparison (Table 2d). The fecundity of females (Table 2e) and numbers of eggs per clutch (Table 2f) did not differ significantly between test (paired with males of the same population) and control groups (paired with males from the other population). Because all control (within-population) pairs from the Czech birch feeding population were kept together, no fecundity data

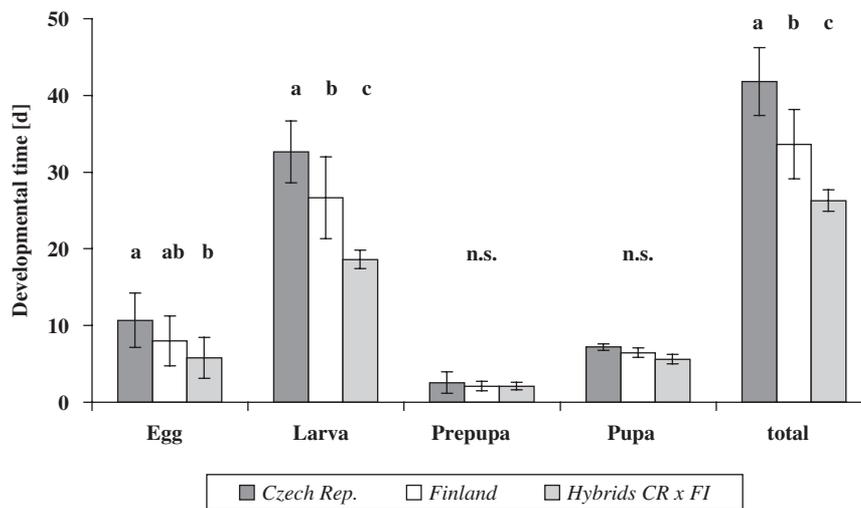


Fig. 2 Developmental times of *C. lapponica* from the Czech (CR) and the Finnish (FI) population, as well as their hybrids (CR x FI), feeding on *Salix fragilis* under same rearing conditions in a climate chamber. Mean values and standard deviations are provided. Czech Republic: egg batches $n=13$, larvae $n=18$, prepupae $n=18$, pupae $n=15$; Finland: egg batches $n=7$, larvae $n=53$, prepupae $n=45$, pupae $n=43$. F₁ hybrids: egg batches $n=2$, larvae $n=56$, prepupae $n=33$, pupae $n=33$. The total developmental times are calculated without the egg stage. Mann-Whitney U-test (Bonferroni-corrected): different letters indicate significant ($p < 0.017$) differences. Data of the parental populations from Gross *et al.* (2004a)

were available. Instead, data from a previous investigation (Gross *et al.* 2004a) were taken into statistical comparison.

Larval survival

The attempt to rear the F₁ hybrid larvae in a no choice experiment on birch leaves failed (Table 3 a). The larvae consumed only trace amounts of leaf material and died during the first larval stage, just as the willow specialized beetle larvae did, when fed with birch (Gross *et al.* 2004a). Only the birch specialized beetles can develop in a no choice experiment on *S. fragilis*, but suffering a very high mortality (97.1 %) (Gross *et al.* 2004a). In contrast, the survival of the hybrids fed with leaves of *S. fragilis* was significantly higher (42.5 %) compared to the survival of both parental generations kept on this willow species (Table 3 a).

Developmental time

The developmental times of specimens of all tested groups (CR x CR, FI x FI, hybrids CR x FI) reared on *S. fragilis* did not significantly differ in the prepupal and pupal stages (Fig. 2). The duration of the egg stage of the F₁ hybrids was shorter than of the parental generations, and differed significantly from the Czech population. Additionally, the larval stage of the hybrids lasted significantly shorter than of larvae from both parental populations, i.e. the total developmental time of the hybrid larvae without the egg stage was about two weeks shorter than that of the Czech larvae and one week shorter than that of the Finnish larvae when fed with *S. fragilis* (Mann-Whitney U-test: $p < 0.017$).

Body weight

Body weights of newly emerged adults, when reared in no choice experiments on *Salix fragilis*, did not differ between the two populations from the Czech Republic (14.21 ± 2.85 mg) and Finland (14.62 ± 2.32 mg). But their F₁-hybrids weighted significantly more (20.47 ± 3.56 mg) than their parents (Fig. 3) (Mann-Whitney U test, $p < 0.001$). But when larvae of both parental populations were reared on their natural host plants, their body weights were significantly higher (Czech population on *B. pubescens* 20.2 ± 2.67 ; Finnish population reared on *S. borealis* 20.3 ± 3.40 mg) (Gross *et al.* 2004a) (Mann-Whitney U-test, $p < 0.001$).

Feeding preferences

Hybrid larvae and adults both clearly preferred willow to birch leaves (Table 3 b). While huge parts of willow leaf disks were eaten, only very small amounts of birch leaf discs were consumed. The differences are statistically significant (Wilcoxon signed rank test for paired samples, $p < 0.001$). The host plant preferences of the F₁ hybrids differ from those of the maternal population, which clearly prefer birch leaves, and are very similar to those of the paternal willow-specialized population (Gross & Hilker 1995).

Discussion

Before we started to interbreed two populations of *C. lapponica*, we tried to evaluate whether the two populations

Table 3. Statistical comparison of survival and feeding preferences between two allopatric populations of *C. lapponica* from the birch-specialized Czech population (CR), the willow-specialized Finnish population (FI), and their hybrids (CR (female) × FI (male)). a) Survival rates [%], χ^2 -test, $p < 0.01$. b) Feeding preferences, Wilcoxon signed rank test for paired samples, $p < 0.01$.

Origin	Czech Republic (CR)	Finland (FI)	F ₁ Hybrid CR×FI
a) Survival			
On <i>B. pubescens</i> (n=24) ¹	30.2 ^b	0 ^a	0 ^a
On <i>S. fragilis</i> (n=56) ¹	2.9 ^a	28.1 ^b	42.5 ^c
On <i>S. borealis</i>	–	83.7 ^d	–
b) Feeding preferences²			
Adults (n=14)	<i>Birch</i>	<i>Willow</i>	Willow
Larvae (n=14)	<i>Birch</i>	<i>Willow</i>	Willow

¹Numbers of hybrid beetles at the beginning of the experiment (neonate larvae to adult stage). ^{a,b,c}Different letters indicate significant differences between columns and rows. Data in italics from Gross et al. 2004a; ²Feeding preferences in italics from Gross & Hilker 1995. All other data this paper.

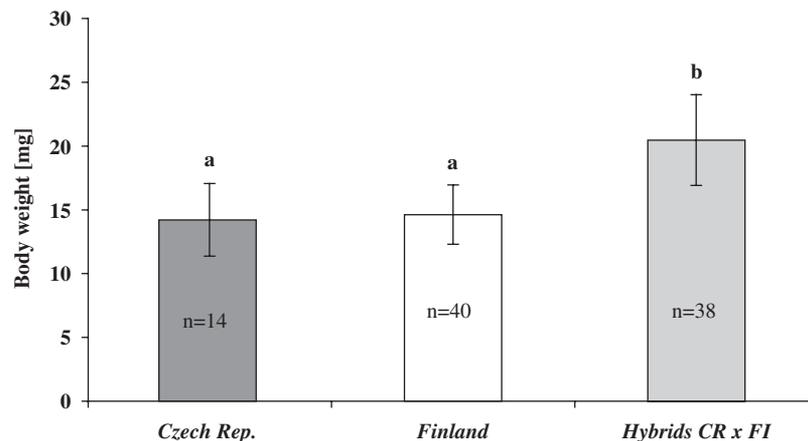


Fig. 3 Body weights of adults of *C. lapponica* from the Czech (CR) and the Finnish (FI) population, as well as their hybrids (CR × FI) reared on *Salix fragilis*. Mean values and standard deviations are given. Mann-Whitney U-test: different letters indicate significant differences ($p < 0.0001$)

were still able to mate and whether the observed courtship and mating behavior between males and females of the same allopecies and in mixed pairs had similar courtship sequences. No differences in the mating behavior between the two investigated populations were found (unpublished results). Jolivet (1999) and Dickinson (1997) studied the sexual behavior among Chrysomelidae and found peculiar mating behavior, often specific and not especially complicated. Interspecific mating was observed between different *Chrysolina* species, but no hybrids were found except for the crosses between *C. herbacea* and *C. coeruleans* (Dickinson 1997; Jolivet 1942; Jolivet 1949; Jolivet 1999). Most of the unnatural mating happens when two species are feeding on the same plant. Mating between a male *Phratora vitellinae* L. and a female of *C. lapponica* was observed on *Salix* in North Lapland (personal observations N. F.). *P. vitellinae* populations use mainly *Salix spp.* as host plants but frequently occur on *Populus spp.* as well (Rowell-Rahier 1984a; Rowell-Rahier 1984b). They were observed in high

densities often occurring on the same plant like *C. lapponica* in Finland (personal observations N. F.). Mating between *Linaeidea aenea* (L.) males and *C. lapponica* females was observed in France (Wüest 1996). In laboratory, mating has been observed very often between different species, genera, or families of Phytophaga. This abnormal behavior might be due to unnatural conditions like overcrowding.

Chemical traits

The chemical composition of the larval secretion of the F₁ hybrids fed with the willow *S. fragilis* showed qualitative differences to the secretions of both the larvae of their mothers fed with birch leaves during larval development and fathers fed with the willow *S. borealis* in the larval stage. In general, main compounds were produced in lower amounts like salicylaldehyde (SA), which content was two times lower in the hybrids' larval secretion. This can be explained

by (a) differences in the content of phenolglycosides like salicin in their host plant's leaves, and (b) maybe by differences in the ability to sequester these host plant components. *S. fragilis* is known to contain less salicin than *S. borealis* (Julkunen-Tiitto 1986; Julkunen-Tiitto 1989). Thus, it is not surprising that the hybrid larvae fed with *S. fragilis* show lower amounts of salicylaldehyde in their secretion that is derived from salicin (Pasteels et al. 1983), than larvae from their paternal population fed with *S. borealis*. Secondly, hybrid larvae might have a reduced ability to sequester salicin from their host plant, and to produce salicylaldehyde. Their birch specialized mothers are known to be able to produce only traces of salicylaldehyde and also salicylalcohol when fed with willow leaves (Gross 1997; Gross and Hilker 1995; Hilker & Schulz 1994) thus indicating that the hybrids might have inherited a reduced ability of salicin sequestration from their mothers.

Morphological traits

Although there is some variation in elytral coloring within the populations of *C. lapponica* (Gross 1997; Milyashevich 2000; Zvereva et al. 2003), the elytra of the Finnish adults are significantly darker than those of the Czech beetles (Gross et al. 2004c). Interestingly, the proportion of the black coloring of the F₁ hybrids of these two populations differs significantly from their parents, being darker than the maternal, but lighter than the paternal elytra. While in some other insect groups variations of specific developmental parameters can cause different phenotypic morphs (Watt 1969), the relationship between black and red color in *C. lapponica* is heritable, which was shown by crossing experiments between different populations from Finland (Zvereva et al. 2003). Our results strongly support these findings. Even though we do not know whether the parents of the hybrids were monozygotic, the phenotype of the F₁ hybrids was intermediate between the phenotypes of the parental specimen. This means that the gene locus for elytral coloring displays an incomplete dominance. The high standard deviations of the parental populations indicated a biological variability of the alleles responsible for darker coloring, but they were more frequent in the Finnish than in the Czech population (Gross et al. 2004c).

Heritable color polymorphism of elytra is also known in the ladybird species *Adalia bipunctata* (Muggleton 1979; Osawa & Nishida 1992). The ecological impact of different elytral colorings in *C. lapponica* was shown by Gross et al. (2004c). When irradiated with light, the darker Finnish adults attained a higher internal body temperature in shorter time than the lighter Czech morphs. Similar results were found for melanic and non-melanic morphs of a ladybird beetle (De Jong et al. 1996). The different elytral coloration of the two all-species is advantageous for *C. lapponica*, because the darker morphs live at lower ambient temperatures (Northern Lapland, Finland) as the lighter ones (Czech Republic) and need to optimize the use of warming radiation (Gross et al. 2004c).

Hybrid fitness advantage and performance traits

Despite the fact that hybrids are most likely not viable under field conditions, we observed some forms of hybrid fitness

advantage for several parameters measured under laboratory conditions. Interestingly, the hybrids performed better on the willow species *S. fragilis*, which is less acceptable for individuals of both parent populations, than their parents. They developed faster, showed lower mortality, and higher adult weights than individuals of both parent populations (Table 3a, Fig. 2, 3). But when larvae of both parental populations developed on their natural host plants, the body weights of the newly emerged adults were significantly higher than those of the hybrids (Gross 2004a; Mann-Whitney U test, $p < 0.001$). In contrast, the developmental time of the maternal population was not affected by their food plant, while the developmental time of the willow specialized paternal population decreased slightly, though significantly, if fed with *S. borealis* (Gross et al. 2004a). But the developmental time of the F₁ hybrids was shorter than that of their parents, irrespective of the plants their parental populations had developed on (Mann-Whitney U-test, $p < 0.001$). Recombinant hybrid genotypes are expected to be, on average, less fit than individuals from the parental species, because most have never been subjected to natural selection (Turelli et al. 2001). However, some hybrid genotypes might be frequently fitter than their parents' (Arnold & Hodges 1995). Such fitness increase may be caused by heterosis, i.e. fitness advantage in outcrossed individuals. Correlation between heterozygosity and fitness components, like developmental stability (Palmer & Strobeck 1986), growth rate (Mitton et al. 1984; Zouros & Foltz 1987), morphological variance (Zink et al. 1985), or resistance to host plant defense (De Jong & Nielsen 2000) has been demonstrated in different species. Hybridization between populations that accumulate different favorable alleles can yield small fitness gains as a result of both introgression of single genes and fixation of recombinant genotypes (Barton 2001). The better performance of the F₁ hybrids of *C. lapponica* on *Salix fragilis* might be explained by heterozygosity of their alleles. Due to the very low population density of the Czech population, an inbreeding depression may reduce the fitness, so the body weight, viability, or developmental time decline.

While there were no differences in female fecundity between all mating groups in which eggs were produced, the eggs from control mating groups (i.e. within-population pairs) showed a significantly higher viability than the eggs from the Czech females mated with Finnish males. But it remains unclear, whether the viability of the eggs was influenced by heritable factors or whether the artificial hibernation of the beetles had caused the lower viability of the hybrid eggs. The beetles used in the Czech control group (CR × CR) were collected after having overwintered under natural conditions, and the egg viability of the other control group (FI × FI) was not detectable because of an infection with molts. Despite this, no eggs were laid in the reciprocal crosses with females of the Finnish population and males of the Czech population. On the one hand, this result could have been caused by a too small number of crossings between Finnish females and Czech males (CR × FI: 5 reproductive pairs out of 21; FI × CR: 0 reproductive pairs out of 7; $p = 0.290$, Fisher's Exact test). On the other hand, an unidirected hybrid formation could have been responsible, due to a reproductive barrier. Studies on the

hybrid formation of two endemic cave dwelling beetles, *Choleva septentrionis cholsatica* and *C. s. sokolowskii* (Coleoptera: Cholevidae), showed that females of mixed pairs had a lower fecundity than females of pairs from the same subspecies (Ipsen 2000). Hybrid formation was only possible between females of *C. s. sokolowskii* and males of *C. s. cholsatica*, but not *vice versa*. It was suggested that this unidirectional hybridization was due to a reproduction barrier between the two subspecies. Interspecific laboratory crosses between *Diabrotica virgifera* and *D. longicornis barberi* (Coleoptera: Chrysomelidae) showed two barriers for hybridization: one involving mating behavior and a post-insemination barrier with reduced egg-viability (Krysan & Guss 1978).

Postzygotic isolation

Our results provide strong evidence for reproductive isolation between the two investigated populations of *C. lapponica*. In this study, we were able to show the possible occurrence of two postzygotic isolation mechanisms, namely zygotic death and hybrid inviability: (1) When willow specialized Finnish females were crossed with birch specialized Czech males, no eggs were laid, what could be caused by zygotic death. Nevertheless it is possible that if a higher number of pairs between Czech males and Finnish females had been investigated, the crossing would have succeeded. (2) Even though hybrid formation occurred in the reciprocal cross between birch specialized Czech females and willow specialized males under laboratory conditions, the resulting hybrid larvae could not survive on the host plant chosen by their mother (birch). Hybrids strongly preferred to feed on their paternal host plant, on which they can survive only (willow). In the field, hybrid larvae would therefore be inviable on birch since they are not mobile enough to search for a suitable host plant in the nearest surrounding.

Host plant shift and speciation process

In the case of *C. lapponica* it is assumed that a host plant shift from *Salix* to *Betula* occurred, before the ancestral species has split into the two new nowadays existing allospecies (Gross *et al.* 2004a; Termonia *et al.* 2001). Although the two host plants hardly resembled in their secondary metabolites, shifts were still possible because of sympatric occurrence. The host shift may have been pushed mainly by escaping from specialist predators and parasitoids on the willows (Gross *et al.* 2004b; Gross & Fatouros 2006; Keese 1997). The allopatric occurrence exists probably since several thousand years (Gross 1997), no gene flow must have taken place between the two populations of *C. lapponica* leading to a possible genetic differentiation among them. Hybrid formation under natural conditions would not have been possible because of the spatial isolation of the different populations. Nevertheless, mating succeeded in laboratory and hybrids were formed. However, only hybrids were produced between birch-feeding females and willow feeding males, but not *vice versa*. While the mother shows a strong oviposition preference for her natural host plant (birch) (Gross *et al.* 2004a), the feeding behavior of

the offspring was determined by their father's (willow). Hence, in our experiments birch specialized Czech females having mated with willow specialized Finnish males oviposited the eggs on plants (birch) on which the new hatching larvae could not survive. If male beetles may be responsible for the feeding behavior of the resulting hybrids in general, no hybrid larvae would survive on the host plant their mother had deposited them on. The same would occur in case that willow-feeding females produced offspring with birch-feeding males. Based on the facts of this study and previous work, we can conclude that a postzygotic isolation exists between the two allospecies of *C. lapponica*. According to the biospecies concept (Mayr 1942; Mayr 1963) and outstanding differences in morphology, ecology, and ethology between most birch-feeding and willow-feeding populations of *C. lapponica* (Gross & Fatouros 2006), the reproductively separated populations may represent two species.

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