Host-associated divergence in sympatric host races of the leaf beetle *Lochmaea capreae*: implications for local adaptation and reproductive isolation

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Ecological specialization is widely recognized as a major determinant of the emergence and maintenance of biodiversity. We studied two critical facets of specialization – local adaptation and habitat choice – in the host races of the leaf beetle *Lochmaea capreae* on willow and birch. Our results revealed that there is asymmetric disruptive selection for host use traits, and host races achieved different adaptive sets of life history traits through association with their host plant. Beetles from each host race exhibited food and oviposition preference for their own host plant. Reciprocal transplant displayed significant variation in host acceptance and performance: all families from the willow race rejected the alternative host plant before initiation of feeding and all died on this host plant. By contrast, all families from the birch race accepted willow for feeding, but they consumed less and performed less well. Intriguingly, families that performed well on birch also performed well on willow, suggesting positive genetic correlation rather than genetic trade-offs. Our results suggest that the major proximal determinant of host specialization in the willow race is the behavioural acceptance of a plant rather than the toxicity of the food resource. However, in the birch race a combination of behavioural host acceptance and performance may play a role in specialization. Our study sheds light on the mechanisms by which divergent host adaptation might influence the evolution of reproductive isolation between herbivorous populations. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **116**, 169–182.

ADDITIONAL KEYWORDS: divergent selection – ecological speciation – insect–plant interactions – reciprocal transplant – trade-offs.

INTRODUCTION

Understanding the patterns of species richness is one of the main goals in ecology and evolution. Phytophagous insects are notably species-rich, making up a large part of the total biodiversity on Earth (Erwin, 1982; Thompson, 1988; May, 1990; Janz, Nylin & Wahlberg, 2005). Therefore, detecting mechanisms behind the outstanding diversification in these groups will go a long way towards understanding global biodiversity (May, 1990; Wilson, 1992; Janz *et al.*, 2005). In recent years a large body of effort has been devoted to achieve this goal (Ballabeni *et al.*, 2003; Rundle & Nosil, 2005), and the results suggest that insect diversification is accelerated by ecological opportunities such as shifting onto new habitats (Simpson, 1953; Feder & Forbes, 2007; Yoder *et al.*, 2010). The close ecological association of such 'host-shifting' phytophagous populations with a particular host plant can be a powerful fuel in the engine of diversification. However, many open questions remain. For instance, why should a species shun most available resources and evolve to exploit just a limited host range? Which types of characters are usually involved during the process of specialization? Under which circumstances are specialized populations likely to diverge and become separate species (Caillaud & Via, 2000; Egan & Funk, 2006)?

Several hypotheses have been suggested regarding the evolution of ecological specialization and how it can facilitate speciation in phytophagous insects.

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One hypothesis is that the evolution of host specificity is driven by genetic trade-offs in adaptation to the available habitats (Rice, 1987; Fry, 1993; Kawecki, 1997). According to the trade-offs hypothesis, loci responsible for genetic variation in fitness across two different habitats exhibit antagonistic pleiotropy such that alleles that increase a given fitness component in one habitat will result in a decrease in the analogous component in the alternative habitat (Kawecki, Barton & Fry, 1997; Ueno *et al.*, 2003). Evidence for such trade-offs is manifested by negative genetic correlations between fitness traits in habitats (Futuyma & Moreno, 1988; Via & Hawthorne, 2002; Ueno *et al.*, 2003). However, most of the empirical studies have failed to find such a negative genetic correlation, which suggests that there may be no fundamental genetic constraint on generalism (reviewed by Joshi & Thompson, 1995; see also Via & Hawthorne, 2002). These results have promoted several alternative hypotheses for the evolution of host specificity at both the genetic and the non-genetic level (Bernays & Graham, 1988; Rausher, 1988; Fry, 1993; Via & Hawthorne, 2002). For example, accumulation of deleterious mutations in genes that are expressed only when occupying rarely used habitats has been proposed as one of the main alternatives at the genetic level (Kawecki, 1997; Kawecki *et al.*, 1997). Non-genetic alternatives suggest that parasitoid/predator defence, escape from interspecific competition or cognitive constraints can be important elements in pushing insect herbivores towards a narrower habitat spectrum and thus to specialization (e.g. Futuyma, Cort & van Noordwijk, 1984; Bernays & Graham, 1988; Via, 1999; Egan & Funk, 2006).

Different types of traits are usually involved during the process of ecological specialization through evolutionary time. Futuyma & Moreno (1988) suggested that behaviour has an important effect on the evolution of divergent adaptation. Host shift and adaptation to novel habitats is often initiated by evolutionary changes in behaviour: when a certain host species is selected over others as food and oviposition substrate (Futuyma, Keese & Scheffer, 1993; Gassmann *et al.*, 2006). Behavioural shifts thus often precede and subsequently drive divergent selection on physiological traits related to the processing of different plant secondary metabolites and morphological traits (Wcislo, 1989; Gassmann *et al.*, 2006). When ecological characters under divergent selection, or those traits that are genetically linked to them, incidentally cause reproductive isolation, then speciation can occur as a by-product of ecological divergence (Mayr, 1963; Caillaud & Via, 2000; Nosil, 2007). This process of speciation through divergent natural selection and adaptation to contrasting environments has been called 'ecological

speciation' (Funk, Filchak & Feder, 2002; Rundle & Nosil, 2005; Schluter, 2001).

Speciation via adaptation to different hosts under sympatric conditions includes first the formation of host races from within panmictic populations and subsequently further suppression of gene flow by the evolution of reproductive isolation barriers between host races (Feder, Chilcote & Bush, 1988; Via, Bouck & Skillman, 2000; Hawthorne & Via, 2001; Dres & Mallet, 2002; Coyne & Orr, 2004; Janz *et al.*, 2005; Sandoval & Nosil, 2005). The evolution of reproductive isolation barriers between populations might be due to either an indirect result of adaptation to heterogeneous environments (Jiggins *et al.*, 2001; Sandoval & Nosil, 2005) or selection against maladaptive host shifting, 'interspecific' matings and the production of less fit hybrid offspring (Servedio & Noor, 2003; Sandoval & Nosil, 2005). For instance, adaptation to different host plants in herbivorous insects can lead to divergence in host preference and if mating occurs on preferred host plants this can create positive assortative mating, thus reducing the opportunity of encountering individuals from different host plants and restricting gene exchange between them (Funk, 1998; Via, 1999; Funk *et al.*, 2002; Coyne & Orr, 2004; Nosil, Vines & Funk, 2005; Sandoval & Nosil, 2005). Furthermore, specialized adaptation to different host plants can reduce the fitness of immigrants from populations adapted to different host plants relative to the residents in a given host plant (Funk, 1998; Via *et al.*, 2000; Sandoval & Nosil, 2005). This process may have a primary effect on gene flow, as it will restrict movement and thus interbreeding between populations from different host plants ('immigrant inviability'; Nosil *et al.*, 2005).

In summary, ecological traits that directly influence both specialized habitat utilization and reproductive isolation are predicted to be of particular importance in many episodes of species divergence in sympatry. However, there are few empirical examples of natural populations in which both the traits under divergent selection and those causing reproductive isolation are known (Schluter, 1996; Craig *et al.*, 1993; Funk, 1998; Via & Hawthorne, 2002).

In this study we investigated two critical facets of host specialization: local adaptation and habitat choice (Ravigne, Dieckmann & Olivieri, 2009), in the leaf beetle *Lochmaea capreae* L. (Coleoptera: Chrysomelidae). This species exhibits sympatric host races on willow (*Salix* spp.) and birch (*Betula* spp.) (Kozhanchikov, 1946; Kreslavsky & Mikheyev, 1994). Based on its sympatric distribution and disparate host plants, *L. capreae* presents an intriguing organism for studying the relationship between host-related selection, ecological specialization and

speciation. Yet although these host races were described almost 70 years ago, pre-dating most discoveries of sympatric host races (Dres & Mallet, 2002), this system has barely been acknowledged in modern speciation research. This is remarkable, as it as late as 2002 was referred to as one of the few examples (Dres & Mallet, 2002) besides the well-studied apple maggot fly and larch budmoth to exhibit true host races without having achieved species status (i.e. still having considerable gene flow). Thus, sympatric speciation via host switching and subsequent specialization seems to occur 'in action'. Unfortunately, since all previous work has been in Russian, it is largely inaccessible to the vast majority of researchers. Furthermore, written in a different scientific era, results are also mostly devoid of statistics so that objective interpretations are problematic. Nevertheless, previous work seems to imply that differentiation on different hosts are associated with a monogenic recessive mutation restricting individuals from the willow race to develop on birch, and this mutation is believed to have spread in sub-populations of *L. capreae*, where willow is the dominant host species (Kreslavsky & Mikheyev, 1994). Although this scenario explains host specialization in the willow race, it does not fully clarify why a host-restricted birch race evolved instead of an 'oligophagous' genotype fully able to utilize both host plants, which would potentially oust the apparently less fit host-restricted willow race. Local adaptation and ecological specialization would provide a mechanism that would facilitate such host-race formation in *L. capreae*.

A first objective of this study was therefore to re-establish *L. capreae* as a model system for sympatric speciation research and bring it into the modern, more rigorous (and more accessible) literature. To do this we first aimed to confirm that host race characteristics in our sympatric German study population are comparable to the situation in Russian populations. Our primary goal, however, was three-fold. (1) To determine the level of specialization and the proximate determinants of specialized adaptation in the host races of *L. capreae* through analysis of their oviposition preference, feeding behaviour and performance on the two host plants. We tested two main hypotheses about ecological specialization: first, that individuals from each host race have a preference for their native host plant; and second, that each host race accepts more and performs better on the native host plant (hereafter 'home' host plant) relative to replicates of the same host race tested on the alternative host plant (hereafter 'away' host plant). We also estimated the relative fitness of immigrants within each host plant relative to residents as another key comparison from the viewpoint of local adaptation (Kawecki & Ebert, 2004). (2) To evaluate genetic variation in host acceptance and performance in the different environments via the examination of family-level variation. The structure of genetic variation for host use exhibits the potential for further evolution of ecological specialization in the host races (Via, 1991). (3) To estimate genetic correlation across two host plants to test whether trade-offs in performance are essential in specialization in this system. We illustrate the importance of both behavioural and performance characters in ecological divergence and how these ecological traits may contribute to the evolution of reproductive isolation in this system.

MATERIAL AND METHODS

STUDY ORGANISM

Lochmaea capreae is a chrysomelid beetle mainly feeding on willow, birch and poplar. In the field, it has one generation per year. Adults emerge from winter diapause in May and begin feeding and mating. Females move among host plants in May and June, laying eggs at the base of twigs or below the surface of the soil near the host plants. Newly hatched larvae migrate on to the leaves to feed and later pupate in the soil. Adult beetles of the new generation appear in August and feed until entering diapause in late summer.

Willow and birch host-associated populations are hypothesized to be host races with about 2% gene flow between them, and previous studies have demonstrated that populations are highly sympatric in western Russia, near Lake Baikal and further east (Kreslavsky & Mikheyev, 1994); yet sympatric populations also seem to extend westward at least to our study populations in the western parts of Germany (K. Reinhold, pers. observ.). In the study populations, assortative mating between races seems weekly developed (Sh. Soudi, K. Reinhold, L. Engqvist, in prep.; see also Kreslavsky & Mikheyev, 1994). Yet initial crossing experiments have revealed signs of substantial hybrid inviability (Sh. Soudi, K. Reinhold, L. Engqvist, in prep.), indicating that in our populations, genetic divergence is already at a quite advanced stage. The willow race feeds mainly on *Salix capreae*, whereas the birch race occurs mainly on *Betula pubescens*. However, both races can potentially feed (and also occasionally occur) on other *Salix* species and *Populus* (Kreslavsky & Mikheyev, 1994). The two main host plants belong to different orders with substantial differences in phytochemistry. Willow leaves contain salicin as the main phenolic component and also other related compounds such as chlorogenic acid, and condensed tannins occur in relatively high concentrations. Birch leaves, by contrast, contain betulin as the main phenolic component (Schulz, Gross

& Hilker, 1997). They also differ substantially in morphological characters: birch is relatively large, tree-like with smooth leaves, while willow is smaller, bush-like and exhibits thicker hairy leaves. Consequently, these two hosts are clearly not equivalent substrates for growth and performance by *L. capreae*. Thus, the two host plants constitute different selective environments for the beetles and may therefore be expected to select for different adaptive characteristics.

ANIMAL COLLECTION AND MAINTENANCE

In May 2013, soon after their emergence from over-wintering sites, overwintered adults of *L. capreae* were collected on birch and willow at two locations in Kottenforst (50.715°N, 7.002°E; 50.671°N, 7.009°E) near Bonn, Germany. Here willow and birch are both relatively common especially at forest edges and early successional forest habitats. Field-collected adults of each population were transferred to the laboratory (Bielefeld University, Germany) and kept individually in single Petri dishes (Ø 5 cm). Abiotic conditions were 25 °C, 70% relative humidity and 18/6-h light–dark cycle, which were maintained throughout the experiments. All individuals were fed with leaves of their natural host plants. To obtain foliage for use throughout the experiments, willow (*Salix caprea*) and birch (*Betula pendula*) were collected in the vicinity of the university campus.

PREFERENCE EXPERIMENTS

ADULT FOOD PREFERENCE

Feeding preferences of field-collected males and females were evaluated through a choice experiment in the laboratory. Twenty-five males and 24 females were selected randomly from each host race and placed into a clean Petri dish (Ø 5 cm) lined with moistened filter paper. Two rectangular leaf discs, one from willow and one from birch, were simultaneously offered to each beetle and they were allowed to choose and feed freely on both host plants for 48 h. Feeding responses were quantified as leaf area consumed after 48 h. After the test, all leaf discs were taped to white paper and scanned using a Canon Scanner (IR 3225N; Canon, Tsukuba, Japan). Leaf area consumed was measured from the digital images using ImageJ v.1.34s software (Wayne S. Rasband, National Institute of Health, Bethesda, MD, USA).

OVIPOSITION PREFERENCE

To determine which of the host plants available in the field are preferred for oviposition, choice experiments were conducted in small plastic containers (15 × 15 × 6 cm). Thirty males and 30 females were chosen randomly from each host race and one male and female from the same host race were placed together in each container with one 10-cm host cutting from each host-plant species. The bottom end of each host cutting was inserted in a small plastic cup filled with water, which held the cutting upright and kept it fresh. The top of each container was covered with mesh, which was secured by elastic bands. As we observed that females occasionally dropped eggs on the ground, we separated the area of each host-plant cutting by placing a rectangular paper under each plastic cup on the bottom of container. The assay lasted 28 days and we recorded the number of eggs on the base of each host plant and paper every second day.

RECIPROCAL TRANSPLANT EXPERIMENTS

To test host acceptance and performance of offspring from each host race on both host plant species, we implemented a split brood family design with offspring from 20 families of birch and willow host races. This yielded four different treatments in a 2 × 2 factorial design based on the host species of origin and the host plant to which the insects were transplanted. Larvae of a given family were produced from the eggs laid by a single mother collected in the field. Thus, paternity was unknown and we had to work under the assumption of having full-sib families (Falconer and Mackay 1996). Females were individually kept in clean Petri dishes, where they were provided with fresh food from their natural host plant and allowed to lay eggs. Oviposition was checked daily and eggs were transferred to clean Petri dishes of 30 mm diameter lined with moistened filter paper. This was carried out to prevent any early larval conditioning effects from exposure to the maternal host plant.

OFFSPRING FOOD ACCEPTANCE

The experiment was performed with 14 larvae from each birch family. These were divided equally into two groups and assigned randomly to either willow or birch according to the experimental design. As we initially observed that the willow race commonly does not accept birch for feeding, we randomly assigned only five larvae on birch and ten larvae on willow. A rectangular leaf disc from the respective host plant was added to the Petri dish and larvae were allowed to feed for 48 h. Feeding response was quantified as described for adult food preference.

OFFSPRING PERFORMANCE

This experiment was conducted to test the relative performance of families from each host race on both host plants. Furthermore, this experiment provides estimates of the average within-population genetic correlation between performance measures across two host plants for each fitness component.

Fourteen newly hatched larvae from the same birch families that were tested for host acceptability were used for the offspring performance test. Newly hatched larvae from each family were divided equally into two groups and then allocated randomly to either of two treatments: half to rearing on willow and half to rearing on birch. We also used 15 larvae from the same willow families and assigned five on birch and ten on willow. Thus, for each host race 20 families were used and the experiment was initiated with 280 larvae from the birch race and 300 from the willow race. Hatchling larvae were reared individually in clean Petri dishes ($\varnothing$ 35 mm) with a sheet of filter paper to ensure adequate moisture and provided with young leaves from host plants every second day.

The four performance components examined were survivorship to adulthood, total development time, pupal weight and relative growth rate (RGR). Total developmental time was defined as the period from the day of hatching to adulthood. Developmental time is potentially an important component of fitness in the field because it determines how long larvae are exposed to predators, parasites and other mortality agents (Rausher, 1984). Pupal weight was measured because adult longevity and fecundity in insects is often correlated with size (Rausher, 1984). RGR, which quantifies mass gained per unit time, was calculated based on an exponential growth model (Bossart, 2003) as RGR = $(\ln W_p - \ln W_1) / D$, where W_p is pupal weight, W_1 is initial larval weight and D is larval duration up to the pupal stage. RGR provides us with information on how well families from each host race can utilize different host plants. All larvae were checked once daily to determine whether a change to the next instar had occurred. All individuals were weighed once on the day of hatching and for the second time on the day of pupation. Every second day, fresh host plant leaves were supplied and accumulated frass was removed.

DATA ANALYSIS

For food preferences, we first calculated a preference index (amount consumed from birch/total amount consumed), and then analysed the differences in food preferences between the two host races by a Mann–Whitney U test. To subsequently assess whether individuals from each race exhibit a preference for a certain host plant, we used the Wilcoxon signed rank test comparing leaf area consumed from each of the host plants. Finally, to examine whether host races differ in the strength of the preference for their native host plant we calculated a second preference index (amount consumed from native host plant/total amount consumed), and differences were analysed using a Mann–Whitney U test. To analyse differences in oviposition preference between the two host races we applied a generalized linear model (GLM) using a quasibinomial error distribution (to correct for overdispersion) and a logit link function. We subsequently used the same steps as outlined above to determine which host plant is preferred by ovipositing females and whether host races differ in the strength of the preference for their native host plant.

To explore the structure of genetic variation in host adaptation within the region from which the beetles were collected, different types of variance analyses were performed at both population (host race) and family level with either the whole or a subset of the data set. By using the whole data set, we addressed whether the two host races differ in their behavioural and physiological response to the different host plants. This would be revealed by a significant population (race) × host interaction. A significant interaction is thus also a first indication of local adaptation in one or both of the host races (Via, 1991; Kawecki & Ebert, 2004). Yet, the interaction itself does not reveal any information on the direction and attributes of the adaptation. Subsequently, we therefore tackled different questions by analysing subsets of the data.

First we addressed the so-called 'home vs. away' criterion, which emphasizes the comparison of each population's host acceptance and fitness across the two host plants. Apart from analysing whether each host race performs better on its 'home' host plant, this analysis also partitions the genotype–host plant interaction between the two host races and within each host race separately. Significant population and family effects propose that the host races and families within each host race differ regarding their ability to accept and utilize host plants. A second way of partitioning the data set allows us to address a further criterion: 'local vs. immigrant', which emphasizes variation in host acceptance and performance of local vs. migrant families within each test host plant. In each host plant the families from each host race (local) are expected to show higher fitness than those from the other host race (Kawecki & Ebert, 2004).

Host acceptance and performance analyses were performed using linear mixed-effects models (LMMs), employing restricted maximum likelihood (REML).

The binomially distributed response variable survivorship (death during development = 0, survival to adult = 1) was analysed with generalized linear mixed effects models (GLMMs) using logit as a link factor and employing the lme4 package in R (Bates *et al.*, 2014).

We were not able to determine sex for those individuals that died during development before reaching adulthood. In some analyses, we thus have a missing value issue regarding the predictor sex. For individuals that died during the pupal stage, we have information about pupal weight but no sex information, and these represent nearly 10% of the data set. For host acceptance we have data for all individuals but unknown sex for nearly half of them as they died before reaching adulthood. Thus, we were facing a dilemma by either controlling for the potential effect of sex, yet restricting ourselves to a reduced data set, or using the complete data but ignoring sex effects. To solve this problem we conducted two complementary analyses: first with a dataset restricted to those individuals that completed their development successfully until adulthood and controlling for sex effects (complete case analysis); second, we used all available data while ignoring sex effects (available case analysis). Nevertheless, the results of these analyses were completely consistent (see Results).

Finally, to estimate cross-environment genetic correlations, corresponding traits of individuals that develop on the two different host plants were considered as separate characters. The cross-environment genetic correlation was estimated using the family mean correlation for birch host race. Family mean correlations are calculated by applying the standard product-moment correlation formula to the family means for each host: $r_m = \text{Cov}(X, Y)/[\text{Var}(X) \cdot \text{Var}(Y)]^{1/2}$, where Cov (X, Y) is the covariance of the family means of the character reared on one host plant and family mean of the same character reared on the other host, and Var (X) and Var (Y) are the variances of family means on birch and on willow, respectively.

All statistical analyses were conducted using the statistical software R 3.0.3 (R Development Core Team, 2011). All tests were two-tailed and the null hypotheses were rejected at $P < 0.05$.

RESULTS

### ADULT FOOD AND OVIPOSITION PREFERENCE

We assessed whether both males and females of *L. capreae* from the willow and birch races differ in feeding and also whether females differ in oviposition preference. The laboratory feeding assay revealed that when individuals from both host races were given a choice, they differed significantly in their food preferences (Mann–Whitney U test – females: $U = 0$, $N_1 = N_2 = 24$, $P < 0.0001$; males: $U = 0$, $N_1 = N_2 = 25$, $P < 0.0001$). Both host races exhibited very clear preferences for their native host plant. All males and females from the willow host race rejected birch leaves entirely and chose willow leaves as food (Wilcoxon test – females: $W = 0$, $N = 24$, $P < 0.0001$; males: $W = 0$, $N = 25$, $P < 0.0001$; Fig. 1). A few individuals from the birch population fed on both plants but seemed to strongly prefer their native host plant for feeding, as the proportion of leaf area consumed by adults was significantly higher on birch than on willow (Wilcoxon test – females: $W = 0$, $N = 24$, $P < 0.0001$; males: $W = 0$, $N = 25$, $P < 0.0001$; Fig. 1). Our results also demonstrated that both males and females from the willow host race displayed significantly stronger preference for their native host plant than the birch host race for birch (Mann–Whitney U test – females: $U = 156$, $N_1 = N_2 = 24$, $P = 0.0002$; males: $U = 225$, $N_1 = N_2 = 25$, $P = 0.005$).

The results of the oviposition preference test show that females from the willow and birch host races differ significantly in their preferences ($F_{1,52} = 163.3$, $P < 0.0001$; Fig. 2). Females from the willow host
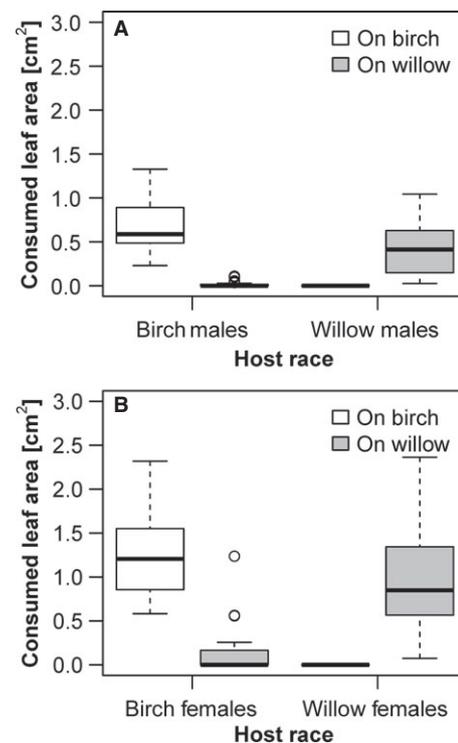
Figure 1. Food preferences expressed by field-collected (A) males and (B) females from both host races of *L. capreae* in laboratory dual choice experiments.
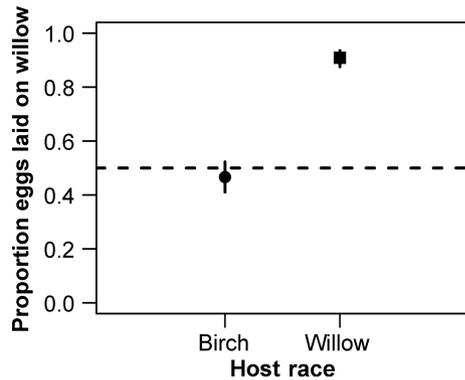
Figure 2. Mean and standard error of proportion of eggs laid by field-collected females from each host race on willow in laboratory dual choice experiments. The dashed line signifies the predictions given an equal distribution of eggs across host plants.

race exhibited a clear oviposition preference; the proportion of eggs was significantly higher on their native host plant than on birch (paired t-test: $t_{26} = 15.2$, $P < 0.0001$). By contrast, birch race females did not significantly discriminate between host plants and the proportion of eggs on their own host plant was only slightly higher than on willow ($t_{26} = 1.02$, $P = 0.31$; Fig. 2). We found a significant difference between the willow and birch races in their oviposition preference for their native host plants, with willow race females exhibiting a significantly stronger preference for their native host plant than birch females ($F_{1,52} = 124.4$, $P < 0.0001$).

OFFSPRING HOST ACCEPTANCE AND PERFORMANCE

Variation in host acceptance and survival between host races

Host acceptance and survival were significantly affected by which host plant was offered and fed to the larvae. Our analysis detected a highly significant population (race) × host interaction for both host acceptance and survival (Table 1). The population × host interaction is especially important because it indicates that one population is more

adapted than the other in using particular host plants (Tilmon, Wood & Pesek, 1998).

Variation within host races ('home' vs. 'away')

The willow host race had a complete disposition to feed on their 'home' host plant with not one of the offspring from 20 families being able to feed on birch; thus, all willow offspring died on birch. Consequently, analyses on the effect of host plant on the other fitness traits within the willow host race became redundant and we conducted the analyses just for the birch host race. When the families from the birch race were compared for their 'home' and 'away' host plant, we found that all families assigned to feed on their 'home' host plant consumed more than those on the 'away' host plant. Mixed model analysis revealed significant among-family variation and a genotype–environment interaction (family × host plant) for larval host acceptance in both types of analyses, which suggests that families respond in different ways to the two host plants. When we included sex in the analysis we did not find any significant effect of sex on host acceptance (Table 2, Fig. 3). Survival of the families was also significantly affected by the host plant on which they were reared (Table 2). All birch families displayed higher survival when they were reared on the 'home' host plant than on the 'away' host plant. A significant family main effect was detected in the mixed effect model when analysis was performed for survival, and we did not find a significant family × host effect (Table 2, Fig. 4A). The genetic correlation calculated for survival was negative but not significantly different from zero ($r = -0.23$, $P = 0.33$).

Birch individuals grew significantly faster when reared on the 'home' host plant, compared with those on the 'away' host plant. The mixed model analysis also detected significant main effects of family and sex, but did not detect any significant family × host interaction (Table 2, Fig. 4B). The genetic correlation calculated for RGR was positive and significantly different from zero ($r = 0.9$, $P < 0.001$).

Pupae were significantly heavier when birch individuals were reared on 'home' host plants than those on the 'away' host plant. Yet our mixed-effects model

Table 1. Mixed effect model to compare variation in larval host acceptance and survival between host races of *L. capreae* on two host plants

Source of variation	Host acceptance			Survival		
	d.f.	χ^2	P	d.f.	χ^2	P
Host	1	114.4	< 0.001	1	28.5	< 0.001
Population (race)	1	115.8	< 0.001	1	17.9	< 0.001
Population × host	1	356	< 0.001	1	165.9	< 0.001

Table 2. Mixed effect models to compare variation in larval host acceptance and performance of the birch population after simulated migration to either willow or birch ('home vs. away' comparison)

	d.f.	Available case analyis			Complete case analyis		
		N	χ^2	P	N	χ^2	P
RGR							
Host	1	173	12.9	< 0.001	171	7.4	< 0.01
Family	1		11.5	< 0.001		14.1	< 0.001
Family × host	2		3.0	0.2		3.0	0.2
Sex	1		–	–		13.5	< 0.001
Pupal weight							
Host	1	204	136.9	< 0.001	172	101.4	< 0.001
Family	1		4.5	0.03		3.2	0.06
Family × host	2		2.3	0.3		3.2	0.1
Sex	1		–	–		12.4	< 0.001
Developmental time							
Host	1	176	< 0.01	0.9	170	1.4	0.2
Family	1		19.2	< 0.001		23.1	< 0.001
Family × host	2		4.9	0.08		5.3	0.07
Sex	1		–	–		13.9	< 0.001
Survival							
Host	1	280	29.5	< 0.001	Not applicable		
Family	1		79.0	0.05			
Family × host	2		2.0	0.3			
Sex	1		–	–			
Host acceptance							
Host	1	280	135.5	< 0.001	172	68.5	< 0.001
Family	1		79.0	< 0.001		17.6	< 0.001
Family × host	2		49.2	< 0.001		22.3	< 0.001
Sex	1		–	–		0.001	0.97

Available case analysis refers to an analysis in which we use all available data ignoring the effect of sex due to missing data; complete case analysis refers to an analysis in which we use data with complete information.

failed to detect any variation among families or a family × host interaction (Table 2, Fig. 4C). The genetic correlation calculated for pupal weight was positive and significantly different from zero ($r = 0.74$, $P < 0.001$).

Overall, the rearing host plant did not have an effect on total developmental time but host plant affected families differently within the birch population. We did not find any significant difference in responses of families to host plants, which was detected as a non-significant host × family interaction in both types of analyses (Table 2, Fig. 4D). The mixed model analysis also detected significant main effects of sex. The estimated value for the genetic correlation was positive and significantly different from zero ($r = 0.71$; $P < 0.05$).

Variation on each test plant species ('local vs. immigrant')
As all individuals from the willow population rejected feeding and thus died on birch, we conclude

that on birch residents clearly perform better than migrants, and we did not statistically compare host acceptance and relative performance any further. On willow, 'immigrant' birch host race families performed less efficiently on willow than 'local' willow families, as revealed by significant differences in all performance traits (Table 3, Fig. 4), and also for host acceptance (Table 3, Fig. 4). This significant decrease in larval host acceptance and relative performance for families forced to live on the alternative host reveals the extent of the disadvantage that could be experienced by migrants that move between willow and birch.

DISCUSSION

Previous studies by Kreslavsky & Mikheyev (1994) have suggested that the leaf beetle *L. capreae* has host races on two distinct host plants, willow and birch, which co-occur in sympatry in large portions
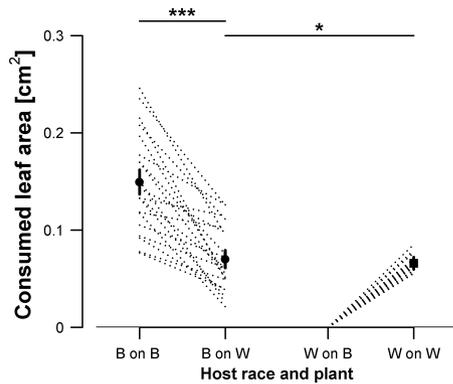
Figure 3. Host × family interaction plots for larval host acceptance of the willow and birch host races of *L. capreae*. Symbols and error bars represent mean estimates ± standard errors for each host plant–race combination. Statistical significance for the different comparisons ('home vs. away' and 'local vs. immigrant') is indicated above the plots: ***P < 0.001, *P < 0.05, n.s. not significant. Dashed lines represent the family-specific norms of reaction. B, birch; W, willow.

of their distribution. Our results partly confirm these results from sympatric populations in the Moscow region of Russia: two distinct host races with clear feeding preferences for their own natal host plant occur in Germany, too. These preferences seem to be asymmetrical, in that there are more distinct preferences within the willow race than within the birch race. Yet, the birch race in our study populations seems to have evolved far more apparent preferences than the corresponding preferences established in Russian populations, where as many as *c.* 30–40% chose willow when given the choice. Intriguingly, sympatric host race formation seems to decline eastward and is nearly absent in eastern Siberia (Kreslavsky & Mikheyev, 1994). Possibly this trend continues towards the west, so that our study population represents a more advanced stage in the sympatric speciation continuum. Alternatively, as the Russian studies were performed some 20–30 beetle generations ago, it is possible that these differences may be an effect of time between studies. The apple maggot fly serves as an illustrative example that the strength of divergent selection may be substantial and cause genetic differentiation also within a relatively short time frame (e.g. Feder, 1998). Our study also confirms an inability of the willow race to develop on birch. Again, this effect is more pronounced in our study population, where no single individual even attempted to feed on birch. Our study also takes this system a step further in that we demonstrate local adaptation in both the willow and the birch races, a process that will potentially facilitate specialization and speciation (see, for example, Ravigne *et al.*, 2009).

LOCAL ADAPTATION, POPULATION DIVERGENCE AND THE SELECTION CONSEQUENCES OF MIGRATION

We studied the main aspects of local adaptation and the selective mechanisms favouring diversification in *L. capreae* by studying behavioural and performance characters, which both typically respond to adaptation and specialization to different host plants (Futuyma *et al.*, 1993).

The two host races of *L. capreae* differ considerably in both host acceptance and performance on the host plants. Differential responses of the host races to the host plants were detected by a significant population (race) × host interaction with crossing reaction norms for both larval host acceptance and survival. This interaction was attributed to a significant reduction in host acceptance and survival of each host race on the 'away' host plant compared with their 'home' host plant. Moreover, the presence of variation in the family × host interaction for host acceptance within the birch race also suggests that there is potential for the evolution of further adaptation in this host race (Via, 1991). Thus, the significant interactions between and within host races provide evidence of local adaptation and indicate that these plant species are very different selective environments for the beetles. Local adaptation according to the 'home vs. away' criterion has repeatedly been reported in other examples of host races such as European corn borer *Ostrinia nubilalis* (Calcagno & Thomas, 2007), and sympatric host races of pea aphids (Via *et al.*, 2000).

The higher fitness of residents relative to individuals that migrate between the host plants provides even stronger evidence of local adaptation. It directly corresponds to divergent selection, which is the driving force of local adaptation and acts on genetic differences in relative fitness between populations within each habitat (Kawecki & Ebert, 2004). The pronounced fitness disadvantage observed when the experimentally migrated families were compared with residents on each host plant indicates that movement between hosts is likely to lead to a significant reduction in fitness. In this beetle mating occurs in spring, so it seems possible that inter-host movement in spring would lead to gene exchange between the host races. However, as migrants will suffer a fitness disadvantage, they will probably comprise a rather small fraction of the gene pool in a given host plant. Thus, the migration potential of beetles may not translate into more than moderate inter-host gene flow. Local adaptation according to such a 'local' vs. 'immigrant' definition has not often been reported
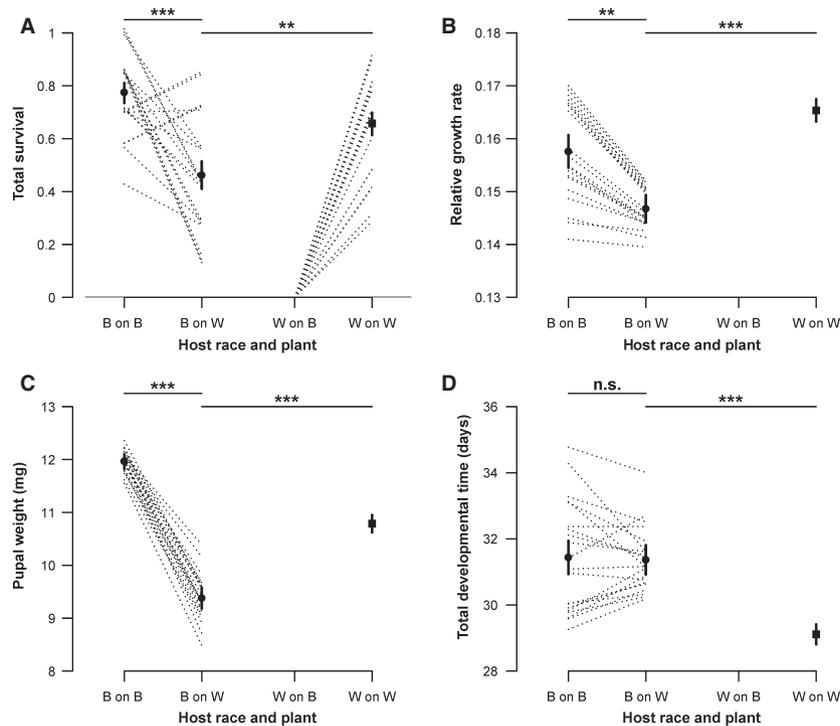
Figure 4. Host × family interaction plots for (A) survival, (B) relative growth rate, (C) pupal weight and (D) total developmental time of the willow and birch host races of *L. capreae*. Symbols and error bars represent mean estimates ± standard errors for each host plant–race combination. Statistical significance for the different comparisons ('home vs. away' and 'local vs. immigrant') is indicated above the plots: ***$P < 0.001$, **$P < 0.01$, n.s. not significant. Dashed lines represent the family-specific norms of reaction. B, birch; W, willow.

(Via, 2001), and in only a few studies have both definitions simultaneously been addressed. Thus, our study provides strong evidence for the evolution of behavioural and physiological adaptation in the host races of *L. capreae* to their host plants.

Selection against individuals that migrate between hosts should result in the evolution of precise host choice behaviour and divergence in host preference by the two host races. The birch and willow races of *L. capreae* differed strongly in their host preferences, yet willow beetles exhibited a much stronger host preference for their native host plant than individuals from the birch race. This asymmetric pattern of host plant preference possibly reflects the outcome of divergent selection on the physiological traits described above. Such an asymmetric pattern was reported in the two species of leaf feeding beetle *Ophraella notulata* and *O. slobodkini* (Keese, 1998). If there was no host preference, we would expect high mortality resulting from inter-host movements and random colonization. Therefore, we expect stronger selection on efficient host choice behaviour in the willow race compared with the birch race. This selection may be strong enough to build up an association between unlinked loci affecting host choice and performance on

each host and leading to speciation (Via *et al.*, 2000). On the other hand, the willow race rejected the alternative host even before feeding began. Thus, one could argue that plant toxicity and nutritional quality are not directly involved in the proximate discrimination among available hosts. In such a case host specialization may be due to behavioural rejection of birch rather than due to physiological adaptation to a plant's chemical defence.

ECOLOGICAL SPECIALIZATION: TRADE-OFFS NOT ESSENTIAL?

Adaptation to different host plants in herbivorous insects through divergent natural selection is expected to lead to trade-offs in performance across environments, a mechanism leading to the evolution of divergent host preference, as individuals shifting host plants are selected against (Nosil, Sandoval & Crespi, 2006). Despite intense investigations for such physiological trade-offs, measured as negative genetic correlations in performance across different habitats, only few have been detected (reviewed by Fry, 1996). Consequently, these findings have led to the speculation that physiological trade-offs are not

Table 3. Mixed effect model analysis to compare variation in host acceptance and performance components of residents and immigrants on willow ('local vs. immigrant' comparison)

	d.f.	Available case analysis			Complete case analysis		
		N	χ^2	P	N	χ^2	P
RGR							
Host race	1	195	25.3	< 0.001	188	23.7	< 0.001
Sex	1	–	–			6.4	0.01
Pupal weight							
Host race	1	216	23.5	< 0.001	188	16.5	< 0.001
Sex	1	–	–			5.3	0.02
Developmental time							
Host race	1	198	16.3	< 0.001	188	15.1	< 0.001
Sex	1	–	–			4.8	0.02
Survival							
Host race	1	340	7.8	< 0.01	Not		
Sex	1	–	–		applicable		
Host acceptance							
Host race	1	340	4.9	0.02	188	4.5	0.03
Sex	1	–	–			0.2	0.6

Available case analysis refers to an analysis in which we use all available data ignoring the effect of sex due to missing data; complete case analysis refers to an analysis in which we use data with complete information.

crucial for the evolution of ecological specialization (Fry, 1996; Kawecki *et al.*, 1997; Caillaud & Via, 2000). Our study also failed to detect any negative genetic correlation in cross-host performance, which indicates that trade-offs in performance traits might not be essential for the evolution of specialization in this study system. Nevertheless, genetic trade-offs in host use may be masked by some environmental effects such as maternal effects. Because we worked under full-sib family assumptions, much of the variation among families and subsequently positive genetic correlation detected in tested fitness components in the birch race could be due to the contribution of maternal effects. Furthermore, failure to detect negative genetic correlations across host plants can be explained by the limitations of statistical methods if genetic covariances are non-negative (Rausher, 1988; Fry, 1993).

Alternatively, the lower fitness of birch population individuals on willow could possibly be explained by the fixation of deleterious mutations (Fry, 1993; Kawecki, 1997), which create higher mutational load and correspondingly lower mean fitness on willow, which is seldomly used as host plant. In fact, this is the mechanism that was originally suggested to promote host specialization in the willow race (Kreslavsky & Mikheyev, 1994). We should also consider possible trade-offs in features other than physiology, such as those in host finding and recognition (Bernays, 2001), or the ability to escape into enemy-free space (Gassmann *et al.*, 2006), which might have played an important role in the evolution and maintenance of specialized adaptation in the birch race.

FROM ECOLOGICAL SPECIALIZATION TO SPECIATION?

Divergent adaptation and specialization on new habitats can act as a first step towards speciation in herbivorous insects (Rice, 1987; Bush, 1994). A probable route to speciation relies on an evolutionary synergism between ecological specialization and reproductive isolation when traits are involved in the two processes. In such a case, divergence as the first step of speciation is accelerated probably because genes subject to divergent selection also pleiotropically affect reproductive isolation (Caillaud & Via, 2000; Servedio *et al.*, 2011).

The host races of *L. capreae* exhibit conspecific populations on two different host plants, and therefore habitat isolation, immigrant inviability and sexual isolation are three potent types of premating isolation barriers that are expected to evolve during the initial phases of divergence between the host races (Nosil *et al.*, 2006). In phytophagous insects mating usually takes place on or very close to the preferred resources (Craig *et al.*, 1993; Via, 1999). Divergence in host preference between populations adapted to different host plants can translate into mate choice, and therefore a system of positive assortative mating will be created, even without any active assessment of potential mates (Via, 1999). Therefore, selection on habitat choice can create a potent pre-mating isolation barrier to gene flow. This has been recently affirmed as the most direct route to sympatric speciation (Servedio *et al.*, 2011), and has been observed in some other examples of host races such as pea aphids (Via, 1999) and apple maggot fly (Bush, 1969; Linn *et al.*, 2003).

However, it is worth noting that if some individuals migrate between host plants complete reproductive isolation between the host races would be expected only if there is direct selection against migrants before mating or if there is selection against hybrid progeny (Liou & Price, 1994; Via, 1999). In the laboratory we observed that assortative mating is relatively weak when beetles are confined together, and it seems that mate choice experiments provide no support for the idea that individuals from a migrant lineage are discriminated against as mates if they are encountered. Strong selection against migrants can further reduce encounters between individuals of the alternative host races and increase assortative mating, thus acting as an additional pre-

mating barrier to gene flow between the host races (Servedio *et al.*, 2011).

CONCLUSIONS

Our findings have implications for sympatric speciation, which has been proposed as the purest form of ecological speciation (Feder & Forbes, 2007). The host races we examined meet the assumptions of proposed verbal and mathematical models of sympatric speciation. We found strong evidence of divergent selection for host use in the host races and the habitat-based preference (Bush, 1969; Turelli, Barton & Coyne, 2001; Berlocher & Feder, 2002; Kawecki, 2004; Sandoval & Nosil, 2005). Thus, *Lochmaea capreae* seems to offer exceptional opportunities to investigate both genetic divergence between natural populations and different aspects of ecological specialization and its role in promoting reproductive isolation and speciation. Such investigations can potentially open the door to determine the underlying molecular and genetic mechanisms that give rise to local adaptation, genetic divergence and speciation in the absence of any physical barriers to gene flow in nature (Caillaud & Via, 2000).

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