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Strong cryptic prezygotic isolation despite lack of behavioral isolation between sympatric host races of the leaf beetle *Lochmaea capreae*

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One of the major goals in speciation research is to understand which isolation mechanisms form the first barriers to gene flow. This requires examining lineages that are still in the process of divergence or incipient species. Here, we investigate the presence of behavioral and several cryptic barriers between the sympatric willow and birch host races of *Lochmaea capreae.* Behavioral isolation did not have any profound effect on preventing gene flow. Yet despite pairs mating indiscriminately, no offspring were produced from the heterospecific matings between birch females and willow males due to the inability of males to transfer sperm to females. We found evidence for differences in genital morphology that may contribute to failed insemination attempts during copulation. The heterospecific matings between willow females and birch males resulted in viable offspring. Yet fecundity and hatchability was remarkably reduced, which is likely the result of lower efficiency in sperm transportation and storage and lower survival of sperm in the foreign reproductive tract. Our results provide evidence for the contribution of several postmating-prezygotic barriers that predate behavioral isolation and act as primary inhibitors of gene flow in this system. This is a surprising, yet perhaps often overlooked feature of barriers acting early in sympatric speciation process.

KEY WORDS: **Hybridization, mate recognition, sexual selection, speciation, sperm.**

Speciation involves the evolution and maintenance of reproductive barriers between interbreeding populations (Dobzhansky 1937; Mayr 1942). These barriers are divided into those that affect the potential for hybridization (premating isolation), and those that occur after mating (postmating isolation). The latter are further subdivided into prezygotic barriers (those acting between mating and fertilization) and postzygotic barriers (those acting after fertilization) (for review and examples see Coyne and Orr 2004). Detailed knowledge of the nature and importance of isolation barriers is an essential step toward determining the primary factors promoting speciation, and may also help to predict whether those barriers will be maintained even in the face of ongoing gene flow and sympatric hybridization (Coyne and Orr 2004; Scopece et al. 2013).

Despite the focus on allopatric speciation over the last century (Mayr 1942), it is now widely accepted that speciation can be maintained without complete spatial separation (Turelli et al. 2001; Via 2001; Bolnick and Fitzpatrick 2007). In the absence of geographic isolation, the homogenizing effects of gene flow is expected to impede the accumulation of isolation barriers and prevent speciation (Felsenstein 1981). However, empirical and theoretical advances have shown that conspicuous phenotypic differentiation between populations can be maintained despite the presence of gene flow by evolutionary forces that inhibit the breakup of favorable allelic combinations and promote speciation (Turelli et al. 2001; Sobel et al. 2010; Briscoe Runquist et al. 2014). This may be achieved when divergent natural selection in contrasting environments generates isolation barriers, a process

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now referred to as "ecological speciation" (Schluter 2001, 2009).

Behavioral isolation (sometimes called "sexual isolation") is often considered to be one of the primary and most important isolation barrier between existing species (Mayr 1963). This mechanism refers to the interaction between traits in different sexes; typically, one sex (usually male) has a signal that stimulates preferences in conspecific but not heterospecific individuals of the opposite sex (Rundle and Nosil 2005). A number of lines of evidence also suggest that it may often be the strongest barrier to gene flow in sympatric regions where females are subject to the risk of cross-specific mating (Coyne and Orr 2004; Etges et al. 2007; Butlin et al. 2012). Divergent natural selection can create coordinated changes in male mating signals and female preferences, leading to behavioral isolation between populations adapted to different habitats (Boughman 2001; Seehausen et al. 2008). Sexual selection that acts on mate preferences and traits involved in behavioral isolation can also be important to speciation when it works in concert with natural selection via ecology; where adaptation to different local habitats can cause sexual isolation by favoring the evolution of female sexual preferences for male ornaments that signal local adaptation (Ritchie 2007; van Doorn et al. 2009).

Unlike behavioral isolation, those barriers that act between copulation and fertilization have until recently been less explored than others. These barriers sometimes are referred to as "cryptic," because they are difficult to study and usually act late in the reproductive sequence (Coyne and Orr 2004; Nosil and Crespi 2006).Cryptic barriers to fertilization can be the by-product of several phenomena. Examples include poor sperm transfer or storage (Price et al. 2001), low viability of foreign gametes in the female's reproductive tract (Gregory and Howard 1993), failure of fertilization when gametes contact each other (Swanson and Vacquier 2002), competition among ejaculates of conspecific and heterospecific males within a single female (Price et al. 2000), or cryptic female choice of sperm (Eberhard 1996). Adaptive divergence could drive the evolution of such cryptic barriers, for instance by influencing genital structures or physical conditions of gametes (Rundle and Nosil 2005; Nosil and Crespi 2006), yet they can also evolve via several other processes, and divergent natural selection need not be involved.

Here, we focus on willow and birch host races (cf. Drès and Mallet 2002 for a defination) of the leaf beetle *Lochmaea capreae*, which are assumed to have about 2% gene flow per generation, suggesting they are at an intermediate stage along the speciation continuum. Investigating incompletely isolated taxa such as host races of *L. capreae* makes it possible to determine barriers directly involved in the initial stages of divergence. Although it is unclear whether such lineages will go on to become permanent biological species, all species have presumably passed through this initial stage of divergence. Therefore, the study of incipient species or populations that are still in the process of diversification has the potential to provide novel insights into the mechanisms of speciation (Sobel and Streisfeld 2015).

Lochmaea capreae feeds mainly on willow (*Salix capreae*, Salicaceae) and birch (*Betula pendula*, Betulaceae). They seem to have sympatric populations extending over large parts of boreal Euro-Siberia (Kreslavskiy and Mikheyev 1994). Previous results have also demonstrated that there is asymmetric disruptive selection for host use traits, and host races achieved radically different adaptive sets of life-history traits through association with their host plant (Soudi et al. 2015). This pronounced host-associated ecological divergence is accompanied by strong ecologically dependent postmating isolation and weaker signs of hybrid breakdown (Soudi et al. 2016), which indicates that divergent natural selection has played a central role in the evolution of hybrid dysfunction between the host races. In the current study, we use a series of crosses within and between sympatric populations of *L. capreae* to quantify the contribution of behavioral isolation and multiple cryptic barriers to hybridization that may influence the maintenance of divergence between these host races. By dissecting these barriers, it is possible to determine the relative contribution of each and establish how gene flow between populations is prevented as well as indicating which barriers arose first.

Materials and Methods

Adults of *L. capreae* were collected on birch and willow on two locations in Kottenforst (50.715^0N, 7.002^0E; 50.671^0N, 7.009^0E) near Bonn, Germany. Eggs were collected from these females and larvae from these eggs were reared on leaves from their native host plants until adulthood, we refer to these adults as the stock population (for details about breeding protocol see Soudi et al. 2015).

NO-CHOICE MATING EXPERIMENTS

No-choice is the most adequate survey of behavioral isolation in this system, as our observations in the field indicate that individuals are rarely confronted with a choice situation. The experiment was conducted by crossing virgin willow (W) and birch (B) adults from the stock population. Two conspecific (W–W and B–B, with the female always listed first), and two heterospecific mating combinations (W–B and B–W) were made to conduct this experiment. For each mating trial, we put one female and one male from either willow or birch into a single plastic petri dish. By direct observation for one hour, we recorded whether copulation occurred or not in each mating trial (i.e., whether the male inserted his aedeagus into the females body or not), the total number of mating attempts, the number of successful mating attempts, copulation

duration and mating latency. We then estimated mating rate (number of successful mating trials to total number of mating trials) and proportion of successful mating (number of successful mating attempts to total number of mating attempts) for each cross-type.

The extent of sexual isolation between the two host races was assessed using the I_{PSI} coefficient in JMating software (Rolán-Alvarez and Caballero 2000; Carvajal-Rodriguez and Rolan-Alvarez 2006). The I_{PSI} coefficient is a sexual isolation index and is defined for every pair combination as the number of observed pair types divided by the number of expected pair types calculated from the matings (Rolán-Alvarez and Caballero 2000). The I_{PSI} estimates the mate choice coefficient for each type of mating pair, and can take values between –1 and +1; zero indicates random mating, +1 and –1 refer to complete assortative and disassortative mating, respectively. Statistical significance of sexual isolation was determined by bootstrapping 100,000 times in JMating (Carvajal-Rodriguez and Rolan-Alvarez 2006).

We performed all other analyses using R version 3.0.3 (R Development Core Team 2012). We analyzed mating rate and proportion of successful mating by applying a generalized linear model (GLM) with cross-type as a single factor in the *lme4* package (Bates et al. 2013). Variation in mating latency between cross-types was evaluated using ANOVA. We applied Box-Cox transformation to fit the normality assumption. Due to heterogeneity of variances, we fitted a generalized least square model (gls) in *MASS* package (Venables and Ripley 2002) to test the effect of cross-type on copulation duration. Subsequently, differences among cross-types were identified by applying a post-hoc Tukey's HSD test using the *glht* function in the *multcomp* package (Hothorn et al. 2008).

LIFE-TIME FECUNDITY AND PROGENY PRODUCTION

The effect of heterospecific mating on female fecundity and progeny production was determined by using two conspecific (W–W and B–B) and two heterospecific crosses (W–B and B–W). Singly mated females were kept individually in a clean single petri dish for oviposition. Every two days we counted the eggs produced by each female and this was continued until all females had died. The progeny production (number of eggs that hatched) was checked daily, and the number of progeny was recorded for each female.

We analyzed life-time fecundity per female using one-way ANOVA, with cross-type as single factor. Since the heterospecific mating between birch females and willow males (B–W) did not yield any hatched offspring (see Results), including this cross-type in the models for hatched progeny per female and hatching rate would violate any parametric model assumptions, we therefore initially used a nonparametric Kruskal–Wallis ANOVA (followed by Dunn's post-hoc test). Subsequently, we excluded the B–W cross-type from the analyses, allowing us to use parametric statistical models. After excluding this cross-type, progeny per female was analyzed using an ANOVA and hatching rate was analyzed using a quasibinomial GLM.

HATCHING RATE DECLINE OVER TIME

The efficiency of sperm storage or survival can be estimated based on the decline in the proportion of eggs that hatch, with an increasing interval between mating and oviposition (Matute et al. 2009; Matute 2010). To estimate how long a female could retain and use viable sperm when she was mated to a heterospecific versus a conspecific male, we used two conspecific crosses (W–W and B–B) and one heterospecific cross that yielded offspring (W–B) and measured the decline in hatchability over time. To assess whether slopes (i.e., the rate at which hatchability decays along time) differ between cross-types we used a generalized linear-mixed effect model (GLMM) using g*lmer* in the *lme4* package. Hatching rate was treated as a response variable, the interaction between time and cross-type as a predictor, and family number nested within cross-type as a random effect. The time × cross-type-interaction would indicate whether the decline in hatching rate is different between cross-types.

SPERM INSEMINATION SUCCESS, SPERM TRANSFER AND STORAGE

To test whether reduction in hatching success in heterospecific crosses is due to problems with sperm transfer, we examined several aspects of insemination in conspecific and heterospecific crosses. First, we assessed whether copulation resulted in successful sperm transfer or not, and second we quantified the number of sperm cells transferred and stored by dissecting females either immediately, 6, or 24 hours after mating. Willow and birch females were randomly assigned to mate with either a conspecific or a heterospecific male. Each pair was allowed to mate only once, meaning that we removed and discarded the males from the mating vial immediately. For sperm transfer, mated females were immediately stored in Eppendorf tubes at –20°C until dissection, and the number of spermatozoa in the bursa copulatrix was determined. For sperm storage, females were transferred to the freezer either 6 or 24 hours after mating, and we counted the number of sperm in the spermathecae. To count the number of spermatozoa, the reproductive tract of mated females was dissected in one drop of 1 × PBS on a microscope slide under a binocular. Bursa and spermathecae were dissected and transferred to a small glass plate. Sperm from these organs were removed with thin pins. To estimate the number of sperm we used an improved Neubauer Haemocytometer (Marienfield, Germany).

The binomially distributed response variable, sperm insemination success, was analyzed with a GLM. In the heterospecific B–W cross, males failed to transfer any sperm to females (see Results). As we described above including this cross-type in the

analysis of sperm insemination success and sperm transfer would violate any model assumptions. We therefore had to exclude it from this particular analysis. To analyze the effect of cross-type on sperm storage, we applied a one-way ANOVA and only females with sperm in their reproductive tract were included in the analysis. We performed post-hoc Tukey's HSD tests to examine differences among three cross-types.

MORPHOMETRIC MEASUREMENTS OF MALE GENITALIA

Geometric morphometric analysis (Zelditch et al. 2012) was used to estimate whether aedeagus size or shape differ between willow and birch races. Male genitalia were photographed with a Leica DM 2500 binocular and Sony DFW digital video camera. Eleven points along the outline that could be located precisely across all specimens were applied as landmarks. Another 74 points, called sliding semilandmarks, were allowed to slide along the outline in the trajectory that minimize shape changes between specimens and the Procrustes average of all the specimens (Rohlf 2007) (Fig. 3). To eliminate nonshape variation, the digitalized landmark data were normalized for position, orientation, and scale by using TPSRELW. Centroid size, the square root of landmarks from the centroid, was extracted and the data were reduced to a series of relative warp scores. Our 11 landmarks and 74 semilandmarks yielded 75 relative warp scores. We only interpreted RW 1–5 (Table S1), which together explained 78% of the variance in the shape of the genitalia.

The size morphometry of male genitalia was investigated using the centroid size of the genitalia as an estimator by applying *t*-test. Following relative warp analysis, differences between host races were assessed using MANOVA. To verify the repeatability of our morphometric measurements of males' genital size and shape, we conducted another complete round of morphological measurements using a different set of pictures. We employed an LMM-based repeatability approach described by Nakagawa and Schielzeth (2010), estimating variance components by restricted maximum likelihood. To estimate confidence intervals, we used parametric bootstrapping with 1000 iterations, based on a method described by Faraway (2006).

Results

MATING BEHAVIOR OF BEETLES AND SURVEYING BEHAVIORAL ISOLATION

We established 18–25 pairs for each of the conspecific and heterospecific cross-types, and recorded 128 successful conspecific and 113 successful heterospecific matings (Table 1). Almost all mating trials (94.6%) led to copulation, and we did not find any significant difference in mating rate between cross-types ($\chi^2 = 3.22$; $df = 3$; $P = 0.3$). By contrast, the frequency of successful mating attempts exhibited a significant difference among the four cross-types ($\chi^2 = 36.56$; $df = 3$; $P < 0.001$), with the heterospecific W–B cross-type showing a lower proportion successful mating attempts in comparison to the others. The outcome of the no-choice experiment did not yield any significant estimates of sexual isolation and showed approximately random mating between willow and birch races ($I_{PSI} \pm SD = 0.11 \pm 0.07$, $P = 0.12$).

Table 1. Results of the no-choice mating trials from the conspecific (B–B and W–W), and heterospecific (B–W and W–B) crosses between sympatric willow and birch host races of *L. capreae*.

	Cross type			
No-choice mating experiment	B–B	B–W	W–B	W–W
No. of mating trials	18	25	29	21
No. successful mating trials	18	24	26	20
Mating rate	1.0	0.96	0.89	0.95
No. of mating attempts	49	62	84	85
No. of successful mating attempts	48	57	56	80
Proportion of successful mating attempts	0.98	0.92	0.66	0.94

Each cross-type is represented by letters W (willow race) and B (birch race), and in each combination female is denoted by the first letter and male by the second.

We did not find any significant differences in mating latency among all four mating combinations ($F_{3, 94} = 2.22$; $P = 0.09$, Fig. S1A). However cross-type had a significant effect on copulation duration ($F_{3, 94} = 24.7$; $P < 0.001$, Fig. S1B). Copulation duration was significantly longer in conspecific birch crosses (by average about 300 seconds longer) than willow crosses (Tukey HSD test, $P = 0.001$). Copulation in B–W cross-types lasted only about 140 seconds that was markedly shorter than all other cross-types (Tukey HSD test, $P < 0.001$).

LIFE-TIME FECUNDITY AND PROGENY PRODUCTION

Cross-type had a significant effect on female fecundity ($F_{3,50} = 21.8$; $P < 0.001$; Fig. 1A). We found that conspecifically mated females produced significantly more eggs than heterospecifically mated females (Tukey HSD test, $P < 0.01$ for all comparisons; Fig. 1A).

The number of hatched progeny per female differed remarkably among cross-types (Kruskal Wallis $\chi^2 = 41.4$, $df = 3$, $P < 0.001$, Fig. 1A). The heterospecific B–W cross did not yield any offspring, no single egg hatched from this cross-type, thus the number of hatched offspring differed significantly from all the other crosses (Dunn's test: B–B: $P < 0.001$, W–W: $P < 0.001$, W–B: $P = 0.002$). Comparing the three crosses, which produced

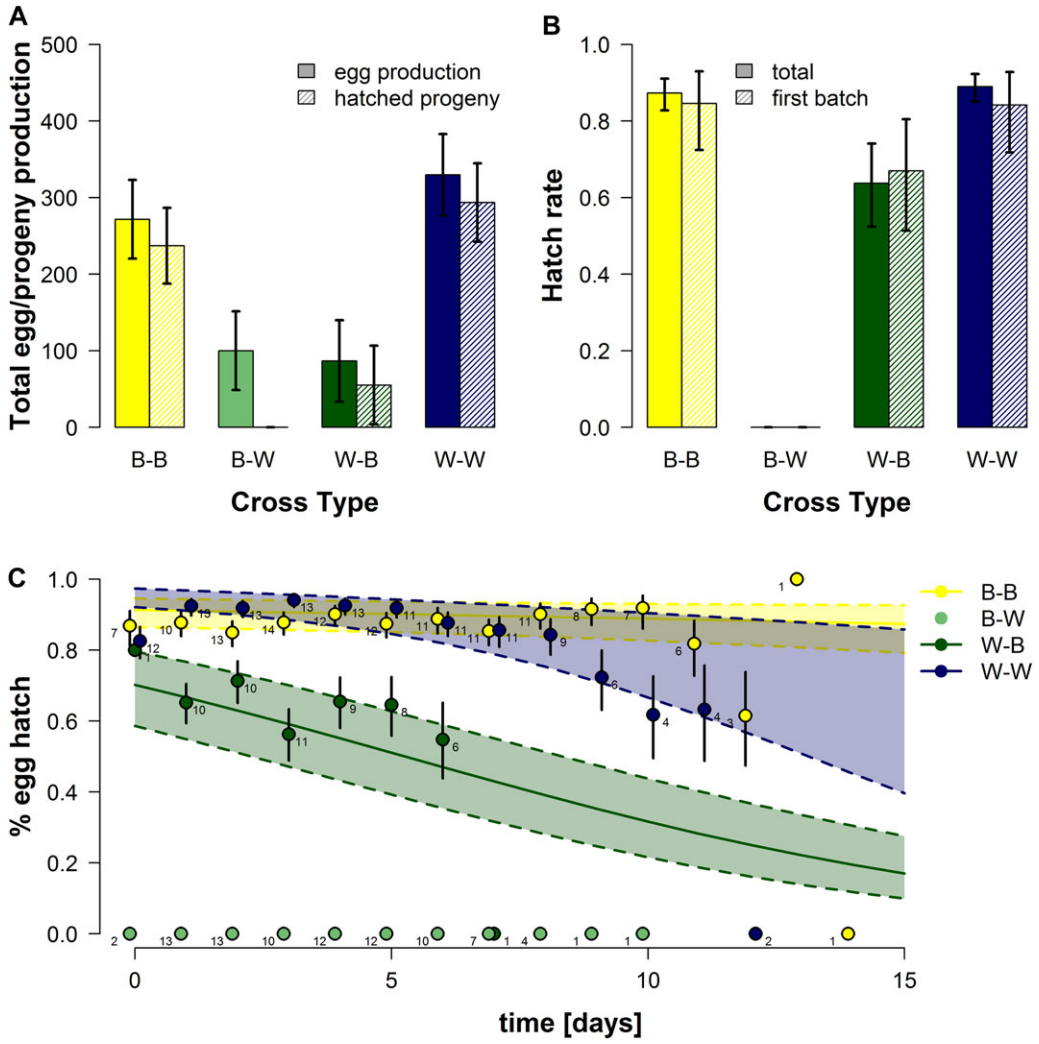

Figure 1. **Mean (±SE) life-time fecundity and progeny production per female (A), total and first batch hatching rate (B), decline in egg hatchability over time (shadowy area is the is the 95% CI from the fitted model, the points represent mean (±SE) estimates at that time point across all families. The numbers beside each point indicate the number of females in each cross-type that we had hatching rate data from at each time point) in conspecific (W–W and B–B) versus heterospecific crosses (B–W and W–B) of *L. capreae*. Each cross-type is represented by letters W (willow race) and B (birch race) with the female parent listed first.**

hatched larvae, we also found significant differences in hatched progeny between crosses ($F_{2,37} = 24.3$; $P < 0.001$, Fig. 1A). This variation reflects the lower progeny production from the heterospecific W–B cross that from the two conspecific crosses (Tukey HSD test, $P < 0.001$). We did not find any significant difference between the two conspecific crosses in the number of produced progeny (Tukey HSD test, $P = 0.26$).

The differences in hatched progeny were also reflected in similar differences in total hatching rate, which varied considerably across cross-types (Kruskal Wallis $\chi^2 = 36.0$, $df = 3$, $P < 0.001$, Fig. 1B). Partly, these differences were caused by the zero hatching rate of all birch females mated to willow males, which differed significantly from the females in all other cross-types (Dunn's test: B–B: $P < 0.001$, W–W: $P < 0.001$, W–B: $P = 0.002$). However, excluding this cross-type also revealed significant differences in total hatching rate between the remaining crosses ($F_{2,35} = 12.5$; $P < 0.001$, Fig. 1B). Specifically, the heterospecific W–B cross differed significantly from the two conspecific crosses (Tukey HSD test, $P < 0.001$). In contrast, there was no significant difference between these three crosses in the hatching rate of the first batch of eggs ($F_{2,35} = 2.33$, $P = 0.11$).

HATCHING RATE DECLINE OVER TIME

There was a clear difference in the reduction of hatching success over time (slope) ($\chi^2 = 28.8$, $df = 2$, $P < 0.001$), with the heterospecific W–B cross-type showing a remarkably stronger decline than the two conspecific crosses (Tukey HSD test, $P < 0.001$; Fig. 1C).

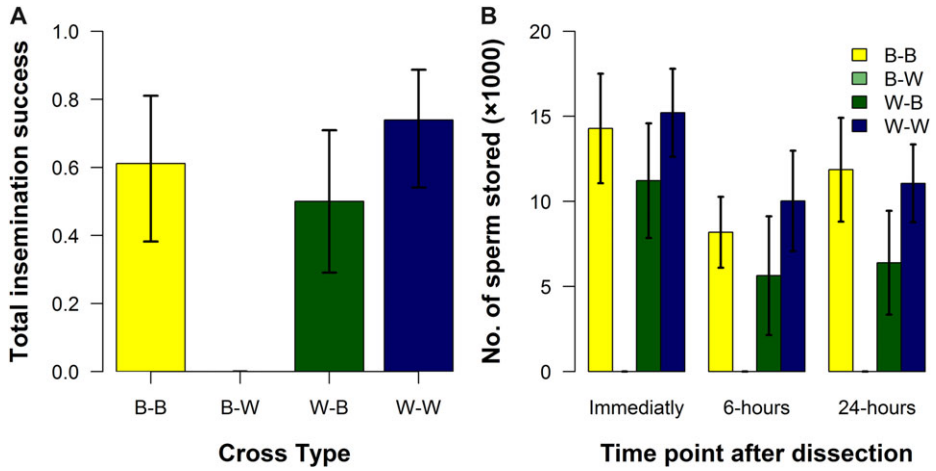

Figure 2. Sperm insemination success (A), and mean (±SE) number of sperm transferred during copulation and stored in spermathecae 6 and 24 hours after mating in successfully inseminated females (B), in conspecific (W–W and B–B) and heterospecific crosses (B–W and W–B) between willow and birch races of *L. capreae*. Each cross-type is represented by letters W (willow race) and B (birch race) with the female parent listed first.

SPERM INSEMINATION SUCCESS, SPERM TRANSFER, AND STORAGE

Sperm insemination success differed markedly among all four cross-types (Kruskal–Wallis $\chi^2 = 34.23$, $df = 3$, $P < 0.001$, Fig. 2A). In the B–W cross-type we found all females with an empty bursa immediately after mating (Fig. 2A). We excluded this cross-type from the subsequent analyses, and ran the model with only the three remaining cross-types. There was no difference between these three crosses in insemination success ($\chi^2 = 2.65$; $df = 2$; $P = 0.2$, Fig. 2A). Further, when we considered only those pairs resulting in successful insemination, we found no differences in number of sperm transferred to females immediately after mating as a measure of insemination success between the three cross-types ($F_{2,35} = 1.86$; $P = 0.17$, Fig. 2B).

The number of sperm stored by females 8 hours after copulation was higher in both conspecific crosses than in the heterospecific cross, although the variation among cross-types was nonsignificant ($F_{2,33} = 1.97$; $P = 0.16$, Fig. 2B). In the 24-h experiment, however, total number of sperm stored in spermathecae was affected by cross-type ($F_{2,31} = 4.11$; $df = 2$ $P = 0.02$, Fig. 2B), with significantly fewer sperm being stored in the heterospecific W–B cross than in the conspecific crosses (Tukey HSD test, $P < 0.001$ for all comparisons; Fig. 2B).

MORPHOMETRIC MEASUREMENTS OF MALE GENITALIA

We did not find any significant difference in genital size between willow and birch males (birch race; 5181 ± 190.54, $n = 36$; willow race; 5115 ± 255.54, $n = 40$; $t = 1.25$, $P = 0.2$). In contrast, genital shape showed an unambiguous association with host race ($F_{5,70} = 18.1$; $P < 0.001$), for both RW1 and RW2, there was a significant effect of host race on the shape of male genitalia. In contrast, for the RW3–RW5 host race did not show any significant effect on the genital shape (Fig. 3, Table S1). The repeated measurements analysis confirmed that our measurements for aedeagus size and shape are also highly repeatable (centroid size: $r = 0.968$, CI: 0.950–0.979; RW1-5: r-values ranged from 0.94–0.47, cf. Table S1).

Discussion

In this study, we investigated the presence of behavioral and several cryptic barriers to gene flow between willow and birch races of *L. capreae*. We found weak behavioral isolation, but significant support for the contribution of several cryptic mechanisms that act substantially during and after mating to reduce gene flow between these host races. Below, we summarize our findings and discuss the evolutionary implications of our findings on reproductive isolation in this system.

NO EVIDENCE OF BEHAVIORAL ISOLATION BETWEEN THE SYMPATRIC HOST RACES OF *L. capreae*

The extent to which males and females contribute to behavioral isolation varies between taxa, although the cost of heterospecific mating is supposed to be higher in females because female investment in each reproductive event is usually expected to be higher than male investment (Kozak et al. 2009). Our results indicate that behavioral isolation is either very limited or absent between these races. These findings stand in contrast to the common notion that behavioral isolation is expected to arise early in the speciation process (Marshall et al. 2002; Chang 2004), and be instrumental in initiating and promoting speciation between

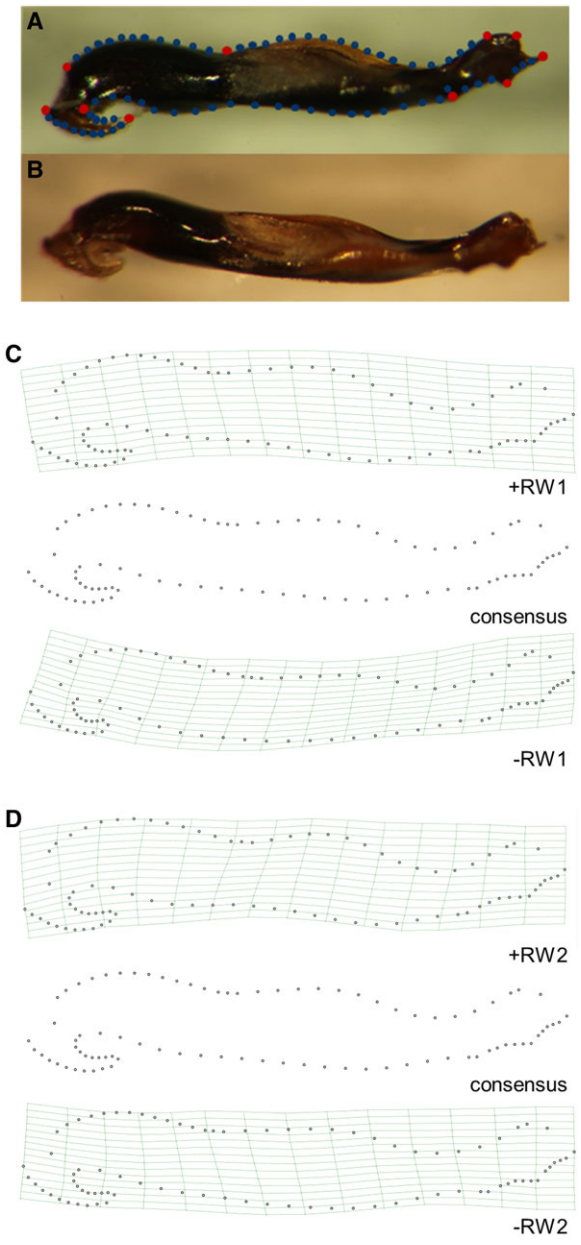

Figure 3. Birch (A) and willow male (B) genitalia of *L. capreae* defined using geometric morphometric approach, which quantified the variation in male genital size and shape. Large red dots on represent Type 2 of landmarks (n = 11), smaller blue dots (n = 74) are sliding semilandmarks. Thin-plate splines of the male aedeagus derived from the relative warp score analysis. Panels show changes in the shape along the first relative warp (C), and second relative warp (D), and the consensus genital configuration (represents the average genital shape).

sympatric taxa (Jiggins et al. 2001; Servedio 2001; Servedio and Noor 2003). The reason for the absence of behavioral isolation remains somewhat puzzling in this system, but it could be explained if preinsemination or prezygotic mechanisms provide females with another opportunity to discriminate against heterospecific males (Veen et al. 2001). Based on the available evidence, male courtship behavior can occur even after the male has already achieved genital coupling, and females may have developed cryptic mechanisms to bias insemination and fertilization in favor of conspecific males (Eberhard 1996b, 2009). Indeed, each of the two hybridizations (B–W and W–B) seems characterized by a unique set of cryptic fertilization barriers, which we discuss in detail below.

BIRCH FEMALES × WILLOW MALES: INSEMINATION FAILURE AND NO HYBRID PRODUCTION

The heterospecific B–W matings did not yield any offspring. None of the birch females mated to willow males had sperm in the bursa and spermathecae when dissected immediately after mating. The lack of sperm in heterospecifically mated birch females seems to be caused by the failure to transfer sperm rather than other mechanism such as expelling sperm caused by females following insemination, a process that has been documented in several insect species (Corderos and Miller 1992; Córdoba-Aguilar et al. 2003). This can act as one of the early barriers to gene flow, which substantially prevents gene flow in the heterospecific crosses between birch females and willow males. Furthermore, we found significant shape differences of the aedeagus between the races. We hypothesize that aedeagus shape might be part of the mate recognition system, providing evidence for a scenario involving cryptic female choice based on the structural/stimulatory faculty conveyed by male genitalia during copulatory courtship (Thornhill 1983; Eberhard 1985, 1996a, 2009). Such pattern of aedeagus shape variation has been suggested to be involved in mate recognition in several other systems like fruit flies (Richmond et al. 2012), or water striders (Bertin and Fairbairn 2005; Rowe and Arnqvist 2012). Alternatively, the lack of sperm in the reproductive tract of heterospecifically mated females can be caused by structural/stimulatory incompatibility between genitalia (i.e., lock-and-key hypothesis) (see Masly 2011 and examples therein). Definite support for the role of genitalia in preventing gene flow in this system, however, requires further investigations.

WILLOW FEMALES × BIRCH MALES: REDUCED SPERM STORAGE AND HYBRID PRODUCTION

The heterospecific W–B matings produced fewer offspring than pure-species pairings. These findings can be explained by lower efficiency in sperm storage, which is conceivably an effective noncompetitive postmating–prezygotic barrier. Similar instances have been recorded in a number of species (Gregory and Howard 1994; Price et al. 2001). Substances in male ejaculates such as seminal fluid proteins have been suggested to play an important function in stimulating female's genital tract to contract and store

sperm in storage organs and therefore contributes to reproductive isolation (Tram and Wolfner 1999; Marshall et al. 2011). These findings may give weight to the idea that differences in the amount or nature of such ejaculate components transferred by birch males might explain why willow females store a smaller fraction of heterospecific sperm. Yet we cannot rule out other possibilities, such as lower sperm mobility in foreign reproductive tract, or even a combination of both female transport and sperm movement in this system.

The impact of sperm storage as part of the female reproductive strategy also depends on how the viability of sperm is maintained in a female's reproductive tract (Neubaum and Wolfner 1998). The decline in egg hatchability over time was significantly heterogeneous between crosses, indicating that sperm stored after mating with a heterospecific male was either retained for a shorter interval or became inviable more quickly than sperm in conspecific matings. Similar results have been reported in matings between two *Drosophila* sister species (Matute and Coyne 2010). Thus, willow females may have the potential to exert cryptic choice by selectively killing stored sperm by several mechanisms such as modifying internal conditions (for instance pH) inside the reproductive tract or inappropriate nourishment, (Eberhard 2009). Therefore, if female choice exists in willow females, it may occur as a consequence of biased sperm transfer, storage and survival inside the female reproductive tract.

Conclusions

Our previous study has shown that there are differences in host preferences between willow and birch races that can translate into habitat isolation (Soudi et al. 2015), and clear evidence for ecologically dependent postzygotic isolation (Soudi et al. 2016). In the present study, we further demonstrate several cryptic isolation barriers that can evolve rapidly and putatively act as the primary barriers to gene flow between these races. Thus, the early stages of speciation in this system proceed via multiple reproductive barriers and not only through divergence in host preferences. Together, these barriers seem to provide an almost complete obstacle to gene flow in this system. The absence of assortative mating by mate choice between these races appears inconsistent with most of the studies that have highlighted the role of behavioral isolation as the primary and stronger impediment to gene flow between sympatric taxa (Coyne and Orr 1989; Wiernasz 1989; Irwin 2002). One possible explanation for the observed pattern is that premating sexual selection (i.e., due to female mate choice) seems generally a weak evolutionary force in this mating system–-almost all mating trials resulted in copulation (Table 1). Conversely, postmating mate discrimination seems significant–-quite a few matings resulted in insemination failure, even some of the conspecific ones (Fig. 2). The absence of any premating sexual selection will eliminate any substrate for behavioral isolation in early diverging lineages and instead expose selection to act on any preexisting cryptic barriers operating during and after copulation. It thus seems likely that the components of reproductive isolation detected in the present study have evolved through the combination of both divergent adaptation and postmating sexual selection. Although, a comprehensive understanding of the evolutionary forces of these barriers will require further studies to determine the ultimate sources and targets of selection in this system.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Bee swarm box-plot for mating latency (a) and copulation duration (b) in conspecific (W-W and B -B) versus heterospecific crosses (B-W and W-B) between willow and birch races of *L. capreae* in no-choice mating experiment. Each cross-type is represented by letters W (willow race) and B (birch race) and in each combination, female is denoted by the first letter, and male by the second.

Table S1. PC-analysis of relative warp scores (RW) and multivariate analysis of variance (MANOVA) table representing the effect of host race (willow and birch) on males' genital shape, as defined by the RW scores.