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When ecological isolation breaks down: sexual isolation is an incomplete barrier to hybridization between *Rhagoletis* species

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ABSTRACT

Question: Environmental disturbance can disrupt habitat choice as an ecological barrier to hybridization between host-specific parasites that mate on their host. Is environment-independent mate choice a sufficient barrier to prevent hybridization when ecological isolation breaks down?

Hypothesis: Males and females will not discriminate between conspecific and heterospecific mating partners in the absence of host cues.

Study system: *Rhagoletis mendax* and *R. zephyria* (Diptera: Tephritidae). Hybridization between these two taxa resulted in the *Lonicera* fly, an example of hybrid speciation in animals. The *Lonicera* fly is found only on non-native honeysuckle. *Rhagoletis mendax* and *R. zephyria* discriminate against each other's host but not honeysuckle. This suggests the local breakdown of reproductive isolation via host choice following the introduction of an invasive plant.

Methods: We combined males and females of both species in a multi-choice experiment in the laboratory and recorded mating events.

Conclusion: Without host plant cues, mate choice is an incomplete barrier to hybridization. Reproductive isolation between host-specific parasites can be influenced by environmental disturbance because a non-ecological barrier (mate choice) alone is too weak to maintain reproductive isolation.

Keywords: hybridization, invasives, mating behaviour, reproductive barrier, sexual isolation, speciation.

INTRODUCTION

Does host choice automatically equate to mate choice in parasites that mate on their host?

Ecological barriers to gene flow play an important role in speciation in general and in the diversification of host specialist parasitic animals in particular (Berlocher and Feder, 2002; Dres and

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Mallet, 2002; Coyne and Orr, 2004). Many host specialists mate on their host (Berlocher and Feder, 2002; Zwölfer, 1974). Host choice is therefore often assumed to equate to mate choice, and the link between these traits is thought to greatly facilitate adaptive speciation following a host shift. Ecological isolating barriers between species are, however, subject to environmental changes and disturbance (Grant and Grant, 2006; Seehausen, 2006). Environmental disturbances that alter host choice behaviours should have a particularly severe effect on the reproductive isolation between host specialist parasites that mate on their host. Alternatively, environment-independent barriers such as assortative mating or post-zygotic sterility are not directly impacted by environmental fluctuations. We must first evaluate the effect of these non-ecological barriers before we can assess the effect of a disturbance on overall reproductive isolation between two species.

Host and habitat choice have been studied extensively as barriers to gene flow in several species complexes of insects in which mating is restricted to the host plant (Berlocher and Feder, 2002; Dres and Mallet, 2002). Much less attention has been paid to the reproductive isolation via assortative mating that is independent of host choice (Zwölfer, 1974; Smith, 1986; Dres and Mallet, 2002; Rodriguez *et al.*, 2004), even though theoretical studies suggest that it plays an important role in completing the speciation process (Johnson *et al.*, 1996). During the early stages of diversification, the assumption that mate choice equals host choice in specialists that mate on the hosts appears to be reasonable. With the important exception of sexual selection, it appears unlikely that individuals within a single population would mate assortatively, unless their behaviour or fitness is directly modified by different hosts (Brazner and Etges, 1993; Wood and Keese, 1990).

Hybrid speciation – a different scenario

The assumption that host choice equals mate choice must, however, be questioned in cases when members of two differentiated populations or species meet on the same host. In this case, the two taxa could be sufficiently differentiated for sexual isolation to have evolved (Johnson *et al.*, 1996). One opportunity for interspecific meetings on the same host are errors in host choice that occur at very low frequencies in the field (Feder *et al.*, 1999). The other possibility is the local breakdown of host fidelity following the introduction of a novel potential host species to the environment that is accepted by more than one parasite species as a resource. One such example of the breakdown of isolation in host-specific parasites due to the introduction of a new host can be found in the colonization of non-native honeysuckle by native tephritid flies in North America. Non-native honeysuckle from the *Lonicera tartarica* species complex (Green, 1966), which was introduced within the past 250 years to North America, is accepted by the two host specialist tephritid fruit flies, *Rhagoletis mendax* and *R. zephyria*, to the same extent as their respective native hosts, blueberry (*Vaccinium corymbosum*) and snowberry (*Symphoricarpos albus laevigatus*). But these two native fly species will not accept each other's native host when they are exposed to blueberry or snowberry in the laboratory. Honeysuckle, blueberry, and snowberry occur in the same landscapes, and it is therefore more likely that *R. mendax* and *R. zephyria* will meet on honeysuckle than on their respective native fruit (Schwarz *et al.*, 2007). We know that successful hybridization between *R. mendax* and *R. zephyria* must have taken place, as non-native honeysuckle is now infested by a new population that initially formed by hybridization between *R. mendax* and *R. zephyria*. Population genetic data demonstrated that this hybrid *Lonicera* fly is now a largely independent population. This system is an

example of homoploid hybrid speciation during which two different species hybridize and give rise to a third species without a change in chromosome number (Schwarz *et al.*, 2005). The specific question for this system is the extent to which sexual isolation represents a barrier for the hybridization of the *Lonicera* fly's parent taxa once the introduction of non-native honeysuckle created a common meeting ground for mating partners from opposite species. The *Lonicera* fly system can also serve as a model for the general question of whether reproductive isolation between host-specific parasites that mate on their host is subject to environmental disturbance. Testing the assumption of whether mate choice equals host choice is crucial in answering this question.

Mating behaviour and sexual isolation in the *Rhagoletis pomonella* species group

Both *R. mendax* and *R. zephyria* belong to the *Rhagoletis pomonella* species group, which consists of several described and undescribed tephritid fruit fly taxa, each of which infests different host plants (Berlocher, 2000). The taxa of the *Rhagoletis pomonella* species group are univoltine fruit parasites that spend their larval life inside the fruit of their respective host plants. All members of the *Rhagoletis pomonella* species group that have been tested to date show high degrees of fidelity to their native host plants (Diehl and Prokopy, 1986; Prokopy *et al.*, 1988; Linn *et al.*, 2005; Schwarz *et al.*, 2007). Compared with what is known about the host fidelity of *Rhagoletis*, little research has been devoted to the mating behaviour of these flies. Field studies established that the site of encounter between the sexes and mating is the host plant in *R. pomonella*. Despite extensive surveys, researchers were unable to locate mating flies off their host plants (Prokopy *et al.*, 1971; Feder *et al.*, 1994). The presence of volatile male sex pheromones has been demonstrated in *R. pomonella*, but it is unclear whether these chemicals play a role in the long-range attraction of females, and host plant cues are regarded as the major mechanism for the first encounters of the sexes (Prokopy, 1975). The courtship behaviour of *Rhagoletis pomonella* (Prokopy and Bush, 1973) and *Rhagoletis mendax* (Smith and Prokopy, 1982) has been described from field observations. Before host fruit has ripened to a stage that is suitable for oviposition, most matings take place on leaves. Most of these matings are initiated by head-to-head encounters (Smith and Prokopy, 1980, 1982). To initiate copulation, a male will jump onto a female. This will either result in successful copulation or the female will try to actively shake off the male. Once fruit is ripe enough for oviposition, most matings occur on the host fruit. In contrast to the early season matings on the leaves, a large proportion of matings is initiated by males mounting ovipositing females (Prokopy and Bush, 1973). These mating attempts tend to be less successful than the attempts that were initiated by head-to-head encounters on the leaves. Successful attempts with ovipositing females are therefore interpreted to consist largely of forced copulations (Smith and Prokopy, 1980). We therefore sought to preserve the greatest degree of female choice by not using artificial oviposition substrates in our experiments. The actual mating system of the *Rhagoletis pomonella* group remains enigmatic, but sexual selection has to be considered as a potential confounding factor in our study of sexual isolation (Jaastad, 1998). Sexual isolation among the host-specific taxa of the *Rhagoletis pomonella* group has received even less attention than the actual mating behaviour. The only study on assortative mating between taxa of the *Rhagoletis pomonella* species group was conducted by Smith (1986). This study uncovered no sexual isolation between the *R. pomonella* and the closely related *Cornus florida* fly (Berlocher, 1999). In contrast, the described taxa *R. mendax* and *R. cornivora* did show significant, if incomplete, sexual isolation from *R. pomonella* (Smith, 1986).

The question of sexual isolation between *R. mendax* and *R. zephyria* is important for reconstructing the initial formation of the *Lonicera* fly in particular and evaluating the potential for hybridization in host-specific parasites in general. Interspecific hybridization in animals is increasingly recognized as an important evolutionary process that results in evolutionary change and gives rise to new diversity (Seehausen, 2004; Arnold, 2006). But the most fundamental condition for evolutionary change via hybridization is that hybrids can form in the first place. Here we present the results of a multi-choice assortative mating experiment in which *R. mendax* and *R. zephyria* individuals were able to choose between conspecific and heterospecific mating partners. Adult flies were not exposed to any host plant cues in order to assess sexual isolation as a non-ecological barrier in the absence of ecological isolation.

MATERIALS AND METHODS

Fly populations and rearing procedure

Adult *R. mendax* and *R. zephyria* were reared from pupae that were collected from infested fruit in the summer and early fall of 2002 following the general rearing procedures described in a previous study (Schwarz *et al.*, 2007). *Rhagoletis mendax* was collected from highbush blueberries at Fennville (Michigan) and East Wareham (Massachusetts). *Rhagoletis zephyria* was collected from snowberry at Munson (Pennsylvania). *Rhagoletis mendax* can be found in wild lowbush blueberries in Pennsylvania, but larval densities were too low to collect sufficient samples for behavioural studies. We cannot exclude geography as a confounding factor for our experiment, but the virtual absence of population structure in *R. mendax* (Berlocher, 1995) supports the notion that observed assortative mating is the result of interspecific difference rather than of local adaptation. Upon eclosion we sorted flies daily by sex to obtain virgin individuals. We used separate climate chambers (22°C and 6 h/18 h dark/light photoperiod) for male and female flies to avoid potential habituation to semiochemicals of the other sex. All cages were washed with soap and rinsed thoroughly with water before re-use. Virgin males and females were allowed to reach sexual maturity (Prokopy *et al.*, 1972) and ranged between 7 and 35 days of age at the time of the experiment.

Experimental procedure

Multi-choice mating experiments with replacement were conducted with 10 females and 20 males each of *R. mendax* and *R. zephyria*. Preliminary trials had shown that the concentration of large numbers of individuals was required to initiate mating in the laboratory. Individuals of the two species were distinguished by a coloured mark of enamel paint (Testors©) that had been applied at least one day before the experiment with a small paintbrush. During the application of paint, flies were not anaesthetized. *Rhagoletis mendax* individuals received a blue and *R. zephyria* flies a red mark. This procedure did not appear to handicap the flies or alter their behaviour. Because fly individuals were limited, we could only conduct one cross and were therefore unable to swap marking colours between the two species. Given our results, however, it would appear unlikely that the different colour markings resulted in a systematic bias in mate choice (i.e. flies with red markings are preferred regardless of their origin or vice versa). The experimental arena was a 1.6-litre rectangular plastic container with multiple entry ports for the easy removal and addition of flies. The arena contained water, food, and two plastic leaves and was placed under six

fluorescent plant lights (P40PL/AQ 40W, General Electric, Louisville, KY) and two greenhouse lights (Lucalox 400W high-pressure sodium bulbs, General Electric, Louisville, KY). The lights provided approximately 4000 lux of light at the centre of the arena, and the temperature at this point was about 30°C. We first introduced the female flies to the cage and allowed them to acclimate for some time before starting the observation by adding the male flies. This ensured that matings did not result from forced copulations with freshly introduced, 'disoriented' females. We surveyed the cages at least every 10 min for copulating pairs. Mating pairs will stay in copula for at least 20 min (Smith and Prokopy, 1982), making it unlikely that successful copulations between individuals were missed. We also recorded unsuccessful mating attempts that consisted of events in which males appeared to be aggressively forcing copulation against the – what we perceived as – visible resistance of a female. During our observations, none of these unsuccessful mating attempts resulted in successful copulation. We removed successfully mating pairs from the experimental arena and replaced them with virgin males and females of the corresponding species to counteract changes in mating propensity that can affect sexual isolation (Casares *et al.*, 1998). No mated individuals were re-tested, but unmated individuals were re-used during consecutive observation periods. This resulted in one long ongoing crossing experiment that was broken into sessions of 2–3 h on consecutive days during the 7th–11th hour of the 16-h light phase of the flies' photoperiod. This window of time corresponds to the greatest mating activity of flies in the field (Prokopy *et al.*, 1972). The entire experiment lasted 16 h 31 min, and a total of 34 females and 37 males of *R. mendax* and 22 females and 39 males of *R. zephyria* were used (the 'pre-mating numbers' for the analysis of sexual selection, see below).

Statistical analysis

We described the sexual isolation between *R. mendax* and *R. zephyria* in our multi-choice experiment with the I_{PSI} estimator of sexual isolation (Rolan-Alvarez and Caballero, 2000). I_{PSI} is calculated from the four estimators of pair sexual isolation (PSI) for each mating combination (the two intraspecific combinations aa and bb and the two heterospecific combinations ab and ba). In each case, PSI is the number of observed mating pairs for a particular combination over the number of matings for this combination that is expected if mating among individuals that actually mated was random. I_{PSI} is the combination of the four different PSI values (Carvajal-Rodriguez and Rolan-Alvarez, 2006):

$$I_{PSI} = \frac{PSI_{aa} + PSI_{bb} - PSI_{ab} - PSI_{ba}}{PSI_{aa} + PSI_{ab} + PSI_{ba} + PSI_{bb}}$$

Like other estimators of sexual isolation, I_{PSI} can take values between −1 and 1. Zero indicates random mating, whereas −1 and 1 indicate that only interspecific or intraspecific matings were observed, respectively. Similarly, indices of asymmetry (IA_{PSI}) can be calculated by taking the ratio of PSI values for the two inter- and intraspecific combinations respectively (Carvajal-Rodriguez and Rolan-Alvarez, 2006). An IA_{PSI} of 1 indicates symmetric mating frequencies between the two mating combinations that were compared. Asymmetry in mating is indicated by IA_{PSI} values that are significantly smaller or greater than 1. Using this framework of estimators has the advantage that we can simultaneously test for the effect of sexual selection in our experiments. Sexual selection can be evaluated by considering the composition of the total population of flies before mating took place versus the population

of flies that actually mated. Pair sexual selection (PSS) is the number of matings for a certain combination *expected* under random mating of flies that actually mated divided by the number of matings for the combination under random mating of the entire pre-mating population. Finally, we also calculated pair total isolation (PTI) indices by dividing the observed number of matings for a given combination by the expected number of matings given random mating among the total pre-mating number of flies (mated and unmated). Using the software JMATING (Carvajal-Rodriguez and Rolan-Alvarez, 2006), we were able to statistically partition the contributions of sexual isolation and sexual selection to the total isolation between *R. mendax* and *R. zephyria* in a *G*-test. We further used bootstrapping (100,000 resamplings) in JMATING to calculate mean bootstrap values, standard deviations (s), and two-tail probabilities for rejecting the null hypotheses that our estimates for PSI, PSS, PTI, and $IA_{\mathbf{PSI}}$ are different from 1 and our estimate of $I_{\mathbf{PSI}}$ is different from 0. When no matings were observed for a particular combination, we changed this zero value to 0.5 to allow for the bootstrap procedure (Carvajal-Rodriguez and Rolan-Alvarez, 2006).

We conducted this analysis twice, first for successful matings and then for the sum of successful matings and unsuccessful attempts. In contrast to successfully mated pairs, the males and females involved in the unsuccessful mating attempts could not be removed from the experimental cage. We could not exclude that unsuccessful attempts altered the behaviour of flies involved such that they were less (or more) likely to be part of another unsuccessful attempt. Unsuccessful mating attempts occurred, however, at low frequency. We therefore neglected the potential effect of experience for the sake of easy comparison and used the same statistics for successful matings and the sum of successful mating and unsuccessful attempts.

RESULTS

Rhagoletis mendax and *R. zephyria* showed substantial but incomplete sexual isolation when only successful mating attempts were considered ($I_{\mathbf{PSI}} = 0.683$, $s = 0.097$, $P < 0.001$, $n = 36$ successful matings). There was a deficiency of successful *R. mendax* female × *R. zephyria* male matings (Fig. 1), and we did not observe any successful *R. zephyria* female × *R. mendax* male matings. The observed asymmetry in the frequency of the two reciprocal interspecific mating combinations was statistically significant ($IA_{\mathbf{PSI}} = 2.974$, $s = 0.759$, $P = 0.009$, $n = 53$ succesful matings and unsuccessful mating attempts), whereas there was no asymmetry between the two classes of intraspecific combinations ($IA_{\mathbf{PSI}} = 0.821$, $s = 0.202$, N.S.). Interestingly, the *R. zephyria* female × *R. mendax* male combination showed the highest number of unsuccessful matings, whereas we did not observe any unsuccessful attempts for the reciprocal interspecific combination. The absence of unsuccessful mating attempts in the latter combination might, however, be a function of the small number of observed matings. In the more frequent intraspecific matings, there was around one unsuccessful mating attempt for every four successful matings (Fig. 1). When we conducted our analysis for the combined number of successful matings and unsuccessful attempts, we still observed significant, but less pronounced, sexual isolation ($I_{\mathbf{PSI}} = 0.398$, $s = 0.131$, $P = 0.004$). In this case, we could not detect any asymmetry for either interspecific ($IA_{\mathbf{PSI}} = 0.923$, $s = 0.253$, N.S.) or intraspecific matings ($IA_{\mathbf{PSI}} = 0.87$, $s = 0.118$, N.S.). Sexual selection does not appear to be a confounding variable as it accounted for only a small fraction of the total isolation (Table 1). The values for PSI, PSS, and PTI that underlie the reported analyses are listed in Table 2.

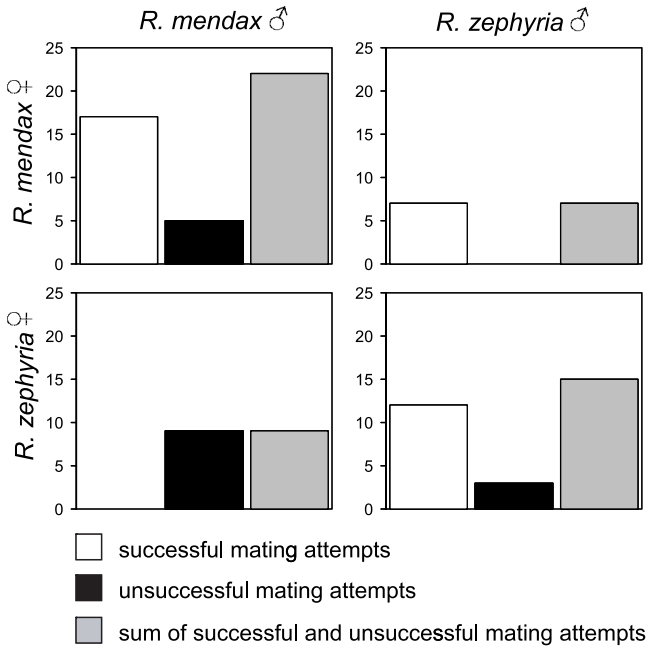

Fig. 1. Observed number of male/female encounters for the four different mating combinations in a multi-choice experiment.

Table 1. Partitioning of the total isolation into the contributions of sexual isolation and sexual selection in a *G*-test (Carvajal-Rodriguez and Rolan-Alvarez, 2006)

	G	d.f.	*P*
Successful matings			
Sexual isolation (GI)	20.82	1	<0.001
Sexual selection (GS)	0.58	2	N.S.
Total isolation (GT)	21.4	3	<0.001
Successful matings + unsuccessful attempts			
Sexual isolation (GI)	8.13	1	0.017
Sexual selection (GS)	2.83	2	N.S.
Total isolation (GT)	10.96	3	0.004

Note: *P*-values were obtained from a chi-square distribution with the degrees of freedom (d.f.) listed in the table.

DISCUSSION

We found that, in the absence of host plant cues, reproductive isolation between *R. mendax* and *R. zephyria* is incomplete. Whereas in laboratory no-choice trials there was no cross-acceptance of each other's host fruit, successful matings between *R. mendax* females and *R. zephyria* males will occur under experimental conditions lacking host plant cues. This

Table 2. Mean bootstrap values for pair sexual isolation (PSI), pair sexual selection (PSS), and pair total isolation (PTI) for each of the four mating combinations

	R. mendax ♂			*R. zephyria* ♂		
	PSI	PSS	PTI	PSI	PSS	PTI
Successful matings						
R. mendax ♀	1.580	1.081	1.597	0.595	1.114	0.625
	(0.553)	(0.261)	(0.282)	(0.265)	(0.254)	(0.211)
R. zephyria ♀	0.099 *	0.870	0.072 *	2.175	0.897	1.654
	(0.172)	(0.322)	(0.101)	(1.344)	(0.316)	(0.388)
Succesful matings + unsuccessful attempts						
R. mendax ♀	1.354	1.084	1.404	0.628	0.729	0.425 *
	(0.379)	(0.217)	(0.229)	(0.305)	(0.185)	(0.149)
R. zephyria ♀	0.681	1.384	0.889	1.658	0.932	1.403
	(0.282)	(0.318)	(0.269)	(0.753)	(0.265)	(0.306)

Note: Standard deviations are listed in parentheses below the observed values. * $P < 0.05$ (100,000 resamplings during bootstrapping).

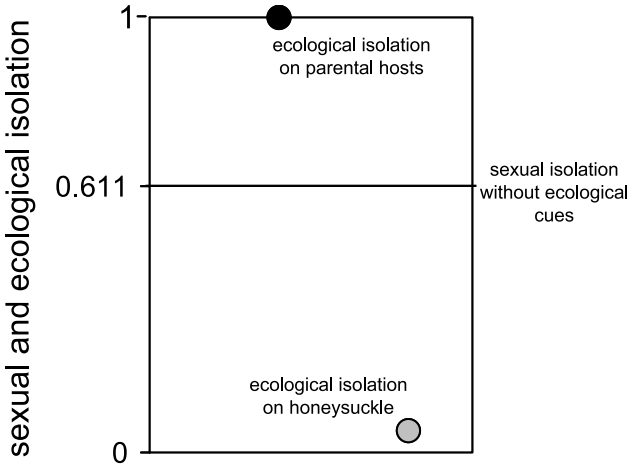

Fig. 2. Comparison of host-independent sexual isolation and ecological isolation via host choice between *R. mendax* and *R. zephyria* on their native hosts and non-native honeysuckle. Sexual isolation is calculated as the joint isolation index (Rolan-Alvarez and Caballero, 2000) from data in this study. Ecological isolation is calculated in an analogous manner using the results of no-choice host acceptance tests from a previous study (Schwarz *et al.*, 2007). An isolation index of 0 indicates random mating or host choice, whereas an index of 1 indicates complete sexual isolation or discrimination of other hosts.

means that when host choice no longer plays a role as a barrier to gene flow because both host specialist insects accept non-native honeysuckle (Schwarz *et al.*, 2007), sexual isolation is an incomplete barrier to hybridization (Fig. 2) that could have resulted in the formation of the *Lonicera* fly (Schwarz *et al.*, 2005). Post-zygotic isolation does not appear to be a complete

reproductive barrier either, as viable hybrids can be produced in the laboratory (D. Schwarz, unpublished).

The adult flies in our experiment did not experience any host cues before or during the experiment, but the tested *R. mendax* and *R. zephyria* individuals spent their larval life in their different respective hosts. Different larval rearing substrates affect the composition of cuticular hydrocarbons in *Drosophila mojavensis*. As these compounds serve as sexual contact pheromones, larval substrate influences sexual isolation between different geographic forms of this species (Brazner and Etges, 1993). We cannot exclude a similar form of larval host conditioning as in *D. mojavensis* (Brazner and Etges, 1993), and the observed sexual isolation of *R. mendax* and *R. zephyria* might have been influenced by different larval substrates. But such conditioning would also occur in nature when *R. mendax* and *R. zephyria* individuals that spent their larval life in their native hosts meet on honeysuckle. Alternatively, honeysuckle could have been colonized by females carrying eggs that had been fertilized by conspecific males on the original hosts. In that case *R. mendax* and *R. zephyria* offspring would have been conditioned in identical larval environments, presumably resulting in less sexual isolation.

Our experiments were conducted in an artificial environment in the absence of any host plant cues. Mating in nature presumably never takes place in the absence of host plant cues, and such cues could interact with mating behaviour. The presence of host materials in mating trials had only a small effect on mating propensity and no consistent effect on sexual isolation in *Neochlamisus bebbianae* leaf beetles that were maintained on their native hosts (Funk, 1998). Keeping adult beetles on non-native hosts before mate choice trials did, however, reduce the sexual isolation to beetle forms that were native to the new substrate (Funk *et al.*, 2002). If such adult stage modification of sexual isolation by host plant cues would also be present in *R. mendax* and *R. zephyria*, we would again expect a reduction and not an increase in interspecific discrimination. We cannot exclude environmental interactions with sexual isolation in our system, but given what is known from other species our experiment will likely overestimate sexual isolation. Either larval or adult conditioning by honeysuckle would likely decrease the strength of sexual isolation as a barrier to interspecific hybridization.

Our experimental design is also artificial in that equal numbers of *R. mendax* and *R. zephyria* individuals were used in our assay. In nature, the spatial distribution of honeysuckle and the two parental hosts could have instead resulted in greatly skewed numbers of inter- and intraspecific mating partners. Despite extensive surveys, we have yet to find a location at which blueberries, snowberries, and honeysuckle co-occur in close proximity. Instead, we find sympatric locations of blueberries and honeysuckle or snowberry and honeysuckle that are connected by the widespread and weedy honeysuckle. It is therefore possible that rare immigrants of one parental species encountered a locally established population of the other parent taxon on honeysuckle. In such a situation, conspecific mates would be rare or absent, a condition that has been shown to decrease sexual isolation in other taxa (Kaneshiro, 1990; Coyne *et al.*, 2005). Finally, flies could also be less selective when matings occur in association with ripe fruit. Matings on fruit often involve copulations with ovipositing females that had been previously interpreted as forced copulations (Smith and Prokopy, 1980).

Sexual isolation between closely related species is often found to be asymmetric (Kaneshiro, 1990), and the results of our study on *R. mendax* and *R. zephyria* corroborate this trend. The observed absence of *R. mendax* male × *R. zephyria* female matings is, however, at odds with

the observed mitochondrial haplotype frequencies in the *Lonicera* fly. DNA-sequence data confirm that the *Lonicera* fly displayed haplotypes from both parent taxa (Schwarz *et al.*, 2005). There are two possible explanations for this apparent contradiction. The first is that *R. mendax* male × *R. zephyria* female matings may occur if there are no conspecific mating partners available (Kaneshiro, 1990; Coyne *et al.*, 2005). This was the case when *R. mendax* males and *R. zephyria* females were confined for a long period in the same cage. This is an anecdotal observation from a cross that was conducted to generate artificial hybrids. The second explanation is the introgression of *R. zephyria* females into a randomly mating population that originated by hybridization between *R. zephyria* males and *R. mendax* females. This population would contain intermediate males (bearing *R. mendax* mtDNA haplotypes) that might successfully mate with *R. zephyria* females.

The asymmetry in sexual isolation disappears when successful matings and unsuccessful attempts are combined for joint analysis. A high number of unsuccessful attempts compensates for the lack of successful *R. mendax* male × *R. zephyria* female matings. The two reciprocal interspecific crosses show similar levels of sexual isolation for the combined data (Fig. 1) even though the overall level of sexual isolation is reduced. This discrepancy suggests that at least two different traits are responsible for the sexual isolation of *R. mendax* and *R. zephyria*. The first trait determines whether encounters between flies are initiated. Its effect can be seen in the sexual isolation that we measured for the sum of successful and unsuccessful mating attempts. The second trait influences whether encounters – once initiated – are successful or result in a higher chance of the male being rejected by the female in an unsuccessful attempt. It is likely responsible for the asymmetry in successful matings and an excess of failed attempts in the *R. zephyria* female × *R. mendax* male combination. Potential mechanisms for sexual isolation could be chemical (Prokopy and Bush, 1972; Prokopy, 1975) or morphological. The shape of the surstylus in the genital apparatus in *R. zephyria* males differs from that observed in other taxa of the *Rhagoletis pomonella* species group (Berlocher, 2000). As the surstyli are likely involved in the coupling of the sexes, this morphological difference could be part of the proximate mechanism of sexual isolation between *R. mendax* and *R. zephyria*.

The only model of sympatric speciation that considers both host choice and sexual isolation in addition to habitat-specific fitness (Johnson *et al.*, 1996) assigns an important role to habitat-independent sexual isolation. While the interplay of host choice and fitness traits leads to the rapid diversification of two populations on different hosts, isolation will not be complete unless selection against maladapted immigrants or host discrimination is complete. The diversification process enters a period of stasis after which it rapidly reaches conclusion once linkage disequilibrium between habitat choice and assortative mating loci develops. Speciation will, however, not be complete unless sexual isolation is error free (Johnson *et al.*, 1996). Under the assumptions of the model, the sexual isolation that we observed in our experiment is insufficient to completely isolate the two parental taxa. This begs the question of whether *R. mendax* and *R. zephyria* can be considered 'good' species. There is, however, experimental evidence that host discrimination and host-specific selection are very strong in the *R. pomonella* species group. Host discrimination between *R. mendax* and *R. zephyria* is complete under laboratory no-choice conditions (Schwarz *et al.*, 2007) and comparisons between laboratory and field experiments in a different species pair suggest that this effect could be even stronger under natural conditions (Diehl and Prokopy, 1986). Population genetic data show that errors of host choice between *R. pomonella* and *R. zephyria* are extremely rare in the field (Feder *et al.*, 1999). *Rhagoletis mendax* and *R. zephyria* are further

differentiated by private alleles at one locus that has been shown to be under strong host-specific selection in *R. pomonella* (Filchak *et al.*, 2000). These reports suggest that complete sexual isolation is not a necessary condition for speciation, as selection and host discrimination are strong enough to effectively isolate described species within the *R. pomonella* species group. Host choice could even lower the effectiveness of sexual isolation in organisms that mate on their host. As errors of host choice are rare, any immigrant fly will not encounter equal proportions of conspecifics and heterospecifics to choose from on different hosts, but will likely find no conspecific mating partners at all, a situation that is expected to reduce sexual isolation (Kaneshiro, 1990; Coyne *et al.*, 2005).

Rhagoletis mendax and *R. zephyria* appear to be almost perfectly isolated species under the environmental conditions in which they evolved. The widespread invasion of non-native honeysuckle was, however, an environmental disturbance that resulted in the complete local breakdown of host fidelity as a reproductive barrier. Sexual isolation alone could not prevent relatively unimpeded hybridization of *R. mendax* and *R. zephyria* on honeysuckle (Fig. 2). These results have interesting consequences for our understanding of the nature of species. They suggest that when ecological barriers are the main mechanism of reproductive isolation, reproductive isolation (and thereby species status according to the biological species concept) can vary in time and space and can be influenced by environmental disturbance (Fig. 2) (Grant and Grant, 2006; Seehausen, 2006). In other examples of the breakdown of ecological isolation in species with incomplete non-ecological isolation, the outcome has been species fusion (Grant and Grant, 2006; Seehausen, 2006). In contrast, the breakdown of isolation between *R. mendax* and *R. zephyria* did not result in the fusion of the species but instead gave rise to a new hybrid origin lineage. This fundamentally different outcome is yet another emergent property of the fact that mating takes place on the host in many host-specific parasites. The different hosts form an ecological mosaic in which the breakdown of reproductive isolation is confined to non-native honeysuckle, which, at the same time, also provides a new resource for hybrid origin flies.

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