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LABORATORY EXPERIMENTS ON SPECIATION

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After neglecting the subject for nearly a century after the publication of *The Origin of Species*, evolutionary biologists have been intensively investigating mechanisms of speciation in the last few decades (reviewed in Barton 2001; Coyne and Orr 2004; Rundle and Nosil 2005; Noor and Feder 2006; Rieseberg and Willis 2007). Experimental evolution approaches have made an important contribution to this resurgence of interest in speciation, complementing theoretical, genetic, and comparative approaches (see also Futuyma and Bennett this volume). This chapter will review the literature on speciation experiments, identify neglected questions that could be addressed by new experiments, and suggest general guidelines for such experiments. Because several reviews of experiments on speciation have been published in recent years (Rice and Hostert 1993; Florin and Ödeen 2002; Kirkpatrick and Ravigné 2002; Coyne and Orr 2004), I will emphasize recent and overlooked experiments, and the prospects and challenges for new experiments.

Although not all definitions of the term *species* explicitly incorporate reproductive isolation (reviewed in Coyne and Orr 2004), whatever definition is adopted, some degree of reproductive isolation is necessary for sympatric species to coexist as distinguishable entities. Hence, understanding the origin of reproductive isolation is necessary for understanding the origin and maintenance of biological diversity. Laboratory experiments on speciation investigate the conditions under which reproductive isolation can evolve between members of what was initially a single, interbreeding population, as well as the conditions under which reproductive isolation between initially partly reproductively isolated populations can become intensified.

The organization of the chapter is as follows. The next section defines terms used in the speciation literature (mostly following Coyne and Orr 2004) and, in so doing, gives an overview of the questions that have been, or could be, addressed by experimental evolution approaches. The second section summarizes the main conclusions from past laboratory experiments on speciation. (I will not address the interesting recent experiments of Rieseberg et al. 1996 and Greig et al. 2002 on homoploid hybrid speciation, a relatively specialized mode of speciation.) The third section suggests questions that have been mostly neglected in past experiments, but which are ripe for further investigation. Finally, the fourth section gives some general guidelines for future laboratory experiments on speciation.

This chapter will focus exclusively on sexually reproducing eukaryotes—that is, those with meiosis and syngamy at some stage of the life cycle. Asexual lineages are automatically reproductively isolated except for occasional horizontal gene transfer (HGT) events. Although bacterial and archaeal lineages form clusters within which HGT occurs relatively easily, but between which HGT rarely occurs (Lawrence 2002), the evolution of such clusters has not, to my knowledge, been investigated experimentally. In experimental microcosms, single bacterial clones may differentiate into multiple, ecologically distinct forms that partition the available environment (Rainey and Travisano 1998; Travisano this volume), but this process does not involve the *de novo* evolution of reproductive isolation.

KEY CONCEPTS

Reproductive isolating barriers can be classified as premating (e.g., lack of response of females to courtship signals of males of another species), postmating but prezygotic (e.g., inability of sperm to fertilize eggs of another species), or postzygotic (e.g., hybrid inviability or sterility, either in the F_1 or later generations). For each type of barrier, it is also useful to distinguish between those that depend on the abiotic or biotic environment ("extrinsic") and those that are relatively independent of the environment ("intrinsic"). For example, two species might form hybrids that are perfectly viable and fertile in the laboratory, but if the species are adapted to different niches, the hybrids would have low fitness in the wild. This is an example of what would be called "ecological isolation" (Coyne and Orr 2004). Ecological isolation can also apply to premating barriers; most notably, species that use different habitats or hosts may mate readily in the laboratory, but will rarely encounter each other in nature.

Reproductive barriers can also be classified by whether they arose in the presence or absence of gene flow between the diverging populations. Allopatric speciation, in which reproductive isolation evolves between geographically separated populations, is accepted as the predominant mode of speciation in most groups, but the possibility of speciation with either no geographic isolation (sympatric) or only partial geographic isolation (parapatric) has gained increasing, albeit far from universal, acceptance in recent years (e.g., Via 2001; but see Coyne and Orr 2004).

A final important set of distinctions involves the evolutionary factors responsible for the origin of reproductive isolation. Most broadly, reproductive isolation could evolve either due to selection or due to random genetic drift (and possibly their interaction); the relative importance of the two has long been controversial. In models that invoke selection, reproductive isolation can evolve either as a by-product of selection on other traits (due either to pleiotropy or linkage disequilibrium) or because of selection for reproductive isolation per se (most notably, in models of reinforcement, in which selection favors avoidance of mating with heterospecifics because of the low fitness of hybrids). Finally, drift can take several forms, such as single bottlenecks, repeated bottlenecks followed by population expansions ("founder-flush" cycles), and extended periods of low population size (see also Futuyma and Bennett this volume).

Thus, evolutionary experiments can be used to investigate such issues as the relative efficacy of natural selection and drift in generating reproductive isolation, the relative rates of evolution of the different types of reproductive barriers, the feasibility of sympatric and parapatric speciation, and the feasibility of reinforcement.

WHAT PAST EXPERIMENTS HAVE TAUGHT US

"Destroy all the hybrids" experiments show that the tendency to mate assortatively can be increased by selection For premating reproductive isolation to evolve between a pair of populations, genetic variation for the tendency to mate assortatively must be present in either or both.

This assumption has been tested in a series of experiments, often called "destroy all the hybrids" experiments, dating back to Koopman (1950). In these experiments, individuals of each sex from two different strains, subspecies, or incompletely reproductively isolated species are placed together and allowed to mate. Some mechanism is then used to ensure that only offspring resulting from "homogamic" (i.e., within-strain) matings are allowed to contribute to the next generation. (Most often, the two strains are homozygous for different recessive genetic markers, so that hybrids between them are immediately recognizable by their wild-type phenotype.) The frequency of homogamic and heterogamic matings is then monitored over successive generations.

I am aware of fourteen such experiments, twelve on flies and one each on maize and yeast, most of which were successful in selecting for increased premating reproductive isolation (table 20.1). Not surprisingly, some of the most rapid responses were in the experiments where the original populations were different species (Koopman 1950; Kessler 1966) or "semispecies" (Dobzhansky et al. 1976). In contrast, all three experiments that reported negative results used strains of *Drosophila melanogaster* as their starting material (of course, it is possible that there were other, unreported negative results). As noted by Coyne and Orr (2004), the lack of response in the experiment of Harper and Lambert (1983) could have been caused by lack of genetic variation in the strains used, which appear to have been "off-the-shelf" marker stocks. In the other two instances where no response was observed, the base populations were outbred, but closely related to one another (Robertson 1966b; Fukatami and Morikami 1970). In these cases, it is possible that there was no initial divergence between the strains in traits affecting mate choice for selection to augment. Nonetheless, the overall conclusion from the experiments summarized in table 20.1 is that there seems to be no shortage of genetic variation for the tendency to mate assortatively in most populations.

An important caveat about these experiments, however, is that they do not lend support to any particular model of speciation. In fact, because hybrids had zero fitness under the experimental regimes (or almost zero; Leu and Murray 2006), the populations were in effect completely reproductively isolated "species" from the outset. Formally, the experiments test for reproductive character displacement—the enhancement of premating isolation between already reproductively isolated species—rather than reinforcement, in which selection against the tendency to hybridize accelerates the speciation process (Butlin 1987). Nonetheless, the rapidity with which reproductive isolation evolved in many of the experiments suggests that experiments to test the reinforcement model might meet with success, though few such experiments have been attempted (discussed later).

Divergent selection in allopatry often leads to the evolution of partial premating isolation The idea that reproductive isolation between allopatric populations often evolves as a by-product of adaptation to different environments has received strong support from several experiments on *Drosophila* and other flies. In these experiments, laboratory populations that had been

TABLE 20.1 Results of "Destroy All the Hybrids" Experiments

Study	Species	Method[a]	Augmented Genetic Variation?[b]	Base Populations Closely Related?[c]	Response[d]
Koopman 1950	*Drosophila persimilis* and *D. pseudoobscura*	VM	Yes	No	+
Kessler 1966	*D. persimilis* and *D. pseudoobscura*	VM	Yes	No	+
Dobzhansky et al. 1976	*D. paulistorum* (two "semispecies")	VM	No	No	+
Wallace 1953	*D. melanogaster*	VM	No	No?	+/−
Knight et al. 1956	*D. melanogaster*	VM	No	No?	+
Robertson 1966b	*D. melanogaster*	DO	Yes	Yes	−
Fukatami and Morikami 1970	*D. melanogaster*	VM	Yes	Yes	−
Ehrman 1971, 1973, 1979	*D. melanogaster*	HL	No	No?	+/−
Crossley 1974	*D. melanogaster*	VM	Yes	Yes	+
Harper and Lambert 1983	*D. melanogaster*	VM	No	No?	−
Hostert 1997	*D. melanogaster*	VM	Yes	Yes	+
Regan et al. 2003	*Musca domestica*	DO	Yes	Yes	+
Paterniani 1969	*Zea mays*	VM	No	No?	+
Leu and Murray 2006	*Saccharomyces cerivisiae*	HL	Yes	Yes	+[e]

[a] VM = visible genetic markers; DO = direct observation of mating pairs; HL = hybrid lethal genetic system.

[b] Indicates whether special steps were taken prior to experiment to ensure that base populations were genetically variable (e.g., by backcrossing marker stocks to diverse wild-type lines).

[c] Indicates whether base populations were derived from the same wild source population.

[d] +, increase in premating isolation observed; −, no increase; +/−, ambiguous or inconsistent results.

[e] This study used a unique design. Evolving experimental populations (E) received immigrants from a reference, nonevolving strain (R) that had been genetically engineered to contain an inducible dominant suicide gene, in a ratio of 10 R cells to 1 E cell. After mating, the suicide gene was induced, killing most but not all R cells and R-E hybrids (approximately 2% of the survivors in the initial generations expressed markers from the R strain). This could be viewed as an experiment to test for reinforcement in a continent-island scenario (see main text's section on "How Readily Does Reinforcement Occur?"), except that the migrants greatly outnumbered residents, the opposite of what would be expected. For this reason, and because of the very strong selection against hybrids, the study is probably better viewed as a test for reproductive character displacement rather than as a test of a realistic reinforcement scenario.

divergently selected for traits as diverse as geotaxis (Soans et al. 1974; Hurd and Eisenberg 1975; Lofdahl et al. 1992), development time (Miyatake and Shimizu 1999), and tolerance of temperature and humidity extremes (Kilias et al. 1980) were found to show significant premating reproductive isolation in mating choice tests (see table 3.1 in Coyne and Orr, 2004, for a summary). In those experiments where two or more replicate lines were selected in the same direction, the replicates usually showed no reproductive isolation from each other, giving powerful evidence that the isolation that had evolved between the divergently selected lines was not the result of genetic drift.

Not surprisingly, not all divergent selection experiments have resulted in reproductive isolation. For example, *D. melanogaster* lines selected in opposite direction for abdominal (Koref-Santibañez and Waddington 1958) and sternopleural (Barker and Cummins 1969) bristle number showed no reproductive isolation, in spite of strong divergence for the selected traits. Thus, some traits apparently show stronger pleiotropic connections to mating behavior than others, although the reasons for the connections are seldom known (but see Miyatake and Shimizu 1999; Rundle et al. 2005). Negative results do not always indicate that the selected trait has no connection to mating behavior, however. In some cases (Mooers et al. 1999; Rundle 2003), the authors did not present evidence that the populations had adapted to their selection regimes. Without such evidence, it is hard to know whether the failure to observe isolation was caused by the choice of trait or by insufficient strength or duration of selection to produce a response. Similarly, in other studies where no reproductive isolation evolved (Robertson 1966b; Markow 1981; also see table 20.2), responses to selection clearly occurred, but selection was unidirectional rather than bidirectional (i.e., selected lines were tested for isolation

TABLE 20.2 Results of "Multiple-Choice" Tests for Premating Isolation among Ethanol-Adapted (HE, for "high ethanol") and Control (R, for "regular food") Populations of Fry et al. (2004)

		Observed Matings (Female × Male)					
Population A	Population B	$A \times A$	$A \times B$	$B \times A$	$B \times B$	Y (S.E.)[a]	X^2
HE1	HE2	5	9	7	6	−0.18 (0.19)	0.90
R1	R2	10	6	7	7	0.13 (0.18)	0.48
HE1	R1	10	8	5	8	0.17 (0.18)	0.88
HE1	R2	9	4	7	5	0.12 (0.21)	0.32
HE2	R1	6	9	7	12	0.03 (0.18)	0.04
HE2	R2	12	7	10	12	0.18 (0.15)	1.28

NOTE: For each replicate, a male and virgin female from each of two populations were placed together in an empty vial; flies from one of the populations were marked by prefeeding with medium containing red food coloring, a treatment shown to have no influence on mate choice in preliminary trials. Vials were placed on their side, and the first mating recorded. Although the sample sizes are low, the HE and R populations show no evidence of premating isolation in spite of their strong divergence in ethanol tolerance (Fry et al. 2004).

[a] Isolation index of Spieth and Ringo (1983); Y significantly > 0 implies assortative mating.

from an unselected base population). Bidirectional selection, by increasing the divergence between the lines, might be expected to be more likely to result in the evolution of reproductive isolation (cf. Florin and Ödeen 2002). (The negative results of Rundle et al. 2003 noted here might also have been due to the authors' use of unidirectional selection, rather than failure of the selected lines to respond to selection in these relatively long-term experiments.)

Of course, even if divergent selection on most traits does not cause reproductive isolation, allopatric populations in nature are likely to be divergently selected for many traits (selection is likely to be "multifarious"; Rice and Hostert 1993). Moreover, such selection takes place on far longer time scales than can be replicated in the laboratory. Thus, even if negative results are underreported, the readiness with which premating reproductive isolation evolves between divergently selected laboratory populations provides strong support for the by-product model of allopatric speciation.

Disruptive selection on arbitrary traits usually does not cause the evolution of premating isolation in sympatry Motivated by the long-standing controversy over sympatric speciation, several sets of investigators have tested whether applying strong disruptive selection to a population can result in the evolution of premating reproductive isolation. In one famous case (Thoday and Gibson 1962), disruptive selection on sternopleural bristle number in *D. melanogaster* resulted in apparently complete assortative mating between the selected extremes within twelve generations. Several attempts to replicate this result with different stocks failed, however. Moreover, similar experiments with different traits and/or species have also usually been unsuccessful at producing reproductive isolation (for reviews, see Thoday and Gibson 1970; Scharloo 1970; Rice and Hostert 1993; Coyne and Orr 2004). The main exceptions to this pattern of negative results have been studies in which the selected trait is either known to be or is at least plausibly related to mate choice—in other words, where a tendency to mate assortatively on the basis of the trait was apparently already present in the base population. Most notably, disruptive selection on geotaxis in the house fly resulted in strong premating isolation (Hurd and Eisenberg 1975). Significantly, divergent selection on this trait in allopatry also caused the evolution of reproductive isolation, as noted above. In contrast, traits used for most of the "unsuccessful" experiments, such as bristle number, apparently had little connection to mate choice, as evidenced in some cases by the failure of divergent selection on these traits in allopatry to produce reproductive isolation (Robertson 1966b; Barker and Cummins 1969).

In general, disruptive selection should lead to selection for any mechanism to reduce mating between the selected extremes, because such matings lead to the production of hybrids with low fitness. In theory, therefore, even if the disruptively selected trait has no effect on mate choice, another trait that affects mate choice and that happens to be fortuitously associated with the selected trait could be recruited to serve as the basis for mating discrimination between the selected extremes. In formal terms, this requires that linkage disequilibrium be present between genes affecting the selected trait and those affecting the mating trait. This "two-trait" or "double-variation" model faces considerable theoretical difficulties, however, because gene flow between the selected extremes and the ensuing recombination continually erode the requisite linkage disequilibrium (Rice and Hostert 1993; Fry 2003). Thus, sympatric speciation is much more plausible when the disruptively selected trait(s) simultaneously serve as the basis of mate choice. This topic is considered in more detail later.

Population bottlenecks, by themselves, seldom lead to the evolution of premating reproductive isolation Some theories of speciation posit a key role for genetic drift (e.g., Mayr 1963; Templeton 1980; Carson and Templeton 1984; for a critical review, see Coyne and Orr 2004). Motivated by these theories, several investigators have subjected populations of *Drosophila* or *Musca* to population bottlenecks and tested whether the bottlenecked lines developed premating isolation from each other and/or from nonbottlenecked control populations (Powell 1978; Dodd and Powell 1985; Ringo et al. 1985; Meffert and Bryant 1991; Galiana et al. 1993; Moya et al. 1995; Rundle et al. 1998; Mooers et al. 1999; Rundle 2003). In most cases, the experiments used multiple single-pair bottlenecks, after each of which the bottlenecked lines were allowed to expand to a large size. Although premating reproductive isolation was observed between some pairs of lines in these experiments, the vast majority of pairwise combinations of lines showed no isolation, and a few even showed negative assortative mating (reviewed in Coyne and Orr 2004). Given the readiness with which reproductive isolation evolves in destroy-all-the-hybrids experiments (table 20.1), the negative results of bottleneck experiments are not likely to have been caused by lack of genetic variation for mating-related traits. Although the relevance of some of these experiments to particular theories of drift-induced speciation can be debated (see especially Templeton 1999 and the reply, Rundle et al. 1999), overall the results indicate that genetic drift, by itself, is only rarely effective at generating premating isolation. Of course, rare events can be important in evolution, so the results do not rule out the possibility that drift-induced speciation sometimes occurs, but they do suggest that this mode of speciation is less common than speciation due to divergent natural selection. Another caveat is that all of the experiments were done on flies; it is possible that bottleneck experiments on other taxa would give different results.

NEGLECTED QUESTIONS

How readily does postmating isolation evolve? Most experimental studies of speciation have focused on premating isolation; we know remarkably little about how quickly postmating isolation can evolve in the laboratory, either due to selection or drift. For example, only five of the twenty or so studies which examined whether divergent selection can lead to premating isolation also reported tests for postmating isolation (see Coyne and Orr 2004, table 3.1). Similarly, most of the studies of bottlenecked lines also failed to report tests for postmating isolation. Of the handful of studies that have tested for postmating isolation, some are uninformative; for example, one simply reported the qualitative observation that F_1 hybrids between bottlenecked lines were fertile (Powell 1978), while another used ill-defined criteria for assessing reproductive isolation (de Oliveira and Cordeiro 1980).

This neglect of postmating isolation in experimental studies is surprising, because there is a widely accepted model by which selection, either alone or in combination with drift, could generate postmating incompatibilities between allopatric populations. The basic idea of the model, which is usually called the Dobzhansky-Muller model (reviewed in Coyne and Orr 2004), is that hybridization is expected to create genetic combinations which have never previously been "tested" by natural selection. Figure 20.1 gives simple examples potentially relevant to experiments in which populations are divergently selected or subjected to bottlenecks. In both cases, the base population is genotype *aabb*, and the mutations *A* and *B* arise and become fixed in one of the descendent populations. In figure 20.1A, *A* and *B* become fixed in different populations; when the populations are hybridized, the two alleles, which have never before been in the same individuals, do not "work" well together, reducing the fitness of hybrids. (The fitness consequences could be manifest in the F_1, as shown in the figure, or delayed until the F_2 or backcross generation, depending on whether the negative interaction between *A* and *B* involves dominant or recessive effects). Although the Dobzhansky-Muller model makes no assumptions about the causes of fixation of the alleles, it is easy to imagine fixation of different alleles in the different populations occurring in response to divergent selection. In figure 20.1B, both *A* and *B* become fixed in the same population; because *A* fixes before *B* arises, however, *B* never occurs together with *a* until the populations hybridize. Figure 20.1B could apply to the case where the first and second populations are selected and control populations, respectively. It might also apply to the situation where the first

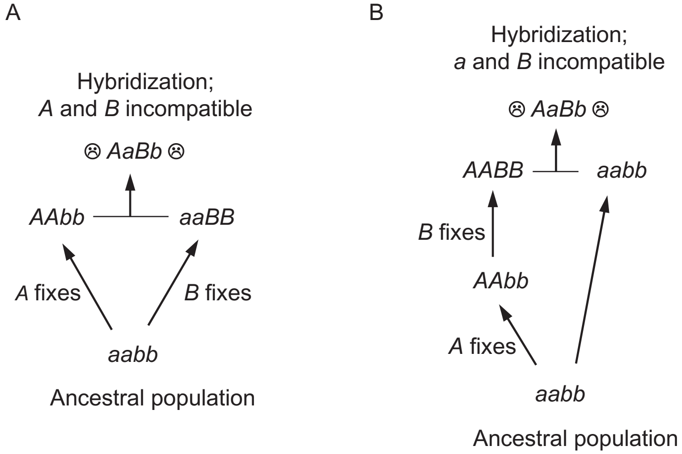

FIGURE 20.1

Dobzhansky-Muller incompatibilities. See text for explanation.

population is subjected to a population bottleneck and then allowed to expand. In this case, the bottleneck could cause fixation of a deleterious allele (*A*) by genetic drift; when the population expands, selection would then be expected to favor modifiers of the deleterious effects of *A* (*B* in the example).

I am aware of only eight studies that give useful data on the evolution of postmating reproductive isolation due to selection in the laboratory, and only a single informative study of the effects of population bottlenecks on postmating isolation. In the latter study (Ringo et al. 1985), eight replicate populations of *Drosophila simulans* were subjected to a series of single-pair bottlenecks, after each of which the populations were allowed to expand. Crosses between the bottlenecked populations and the base population showed a highly significant decline in productivity relative to within-population crosses with successive founder-flush cycles. Because the bottlenecked populations showed no evidence for premating isolation from the base population, the declining productivity of hybrid crosses gives evidence that partial postmating isolation evolved between the lines (either reduced F_1 viability or poor fertilization success in the hybrid matings). Because the base population was produced by crossing flies collected from widely separated localities, however, it is not clear that the incompatibilities arose *de novo*; the bottlenecked lines may have simply segregated out for variants that came from the different localities (cf. Rundle et al. 1998).

Ringo et al. (1985) also selected eight large populations for diverse traits and conducted similar tests of postmating isolation from the base population; none was observed. Kilias et al. (1980) similarly found little evidence for postmating isolation in crosses between a pair of *D. melanogaster* populations adapted to different regimes of temperature, light, and humidity, in spite of strong premating isolation between them. In contrast, Robertson (1966a) and Boake et al. (2003) created lines with different combinations of chromosomes from *D. melanogaster* populations selected for resistance to toxins (EDTA and DDT, respectively) and their respective controls, and they found that one of the "hybrid" chromosome combinations in each case had lower survival and/or fertility under control conditions than either parental population. Because there was only one selected and control population in each study, however, and the chromosome substitution experiments were themselves unreplicated, it is not clear that the selection treatments were responsible for the apparent genetic incompatibilities, if they were incompatibilities at all (e.g., it is possible that one of the parental lines in each study had a high frequency of a deleterious allele by chance, and this became fixed in the chromosome substitution process).

More convincing evidence that divergent selection can rapidly generate postmating isolation comes from an old but neglected study of mites (Overmeer 1966) and two recent studies of fungi (Dettman et al. 2007, 2008). Overmeer (1966), studying the haplodiploid plant pest *Tetranychus urticae*, crossed mites from two populations that had been independently selected for resistance to the pesticide Tedion back to the base population and found that the F_1 hybrids were partly sterile, laying many eggs that did not hatch (figure 20.2). Hybrids between the two selected populations had normal fertility, giving evidence that the isolation from the base population was the result of the resistance selection, not drift. A possible explanation for these results is that the resistance allele(s) initially had negative pleiotropic effects on embryo viability; this would have generated selection for modifiers of these effects in the selection lines. In eggs laid by F_1 females, however (particularly the haploid eggs laid by unfertilized females, which would normally develop into males), the modifiers would have become separated from the resistance alleles by recombination. The existence of negative fitness effects of pesticide resistance alleles, as well as modifiers of those effects, have been demonstrated in at least one other species (McKenzie and Game 1987). Interestingly, Overmeer's results suggest that some of the modifiers were cytoplasmic, because egg hatch was considerably lower when the cytoplasm of the F_1 females came from the base population than when it came from the resistant populations (figure 20.2). In contrast to Overmeer's result, Fry (1999) found no evidence for similar reproductive incompatibility between a *T. urticae* population selected for resistance to a toxic host plant and the control population.

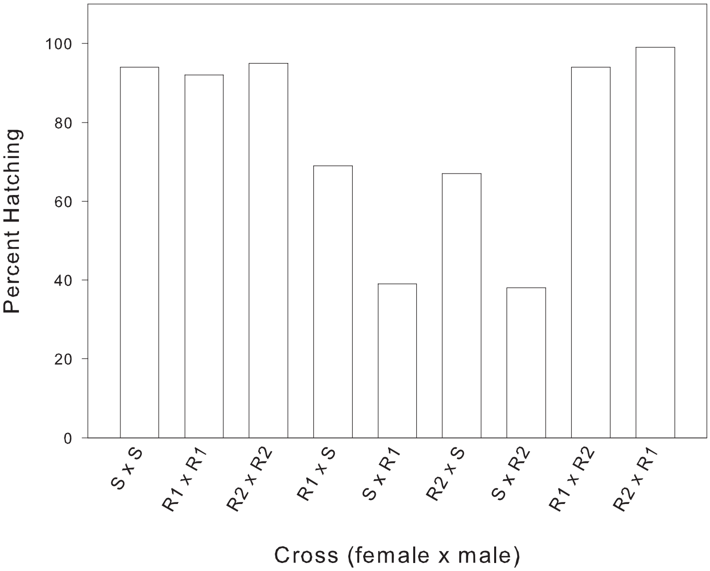

FIGURE 20.2
Hatching rates of haploid (unfertilized) eggs laid by F_1 females from crosses within and between populations of the haplodiploid mite *Tetranychus urticae* either selected for resistance to the acaracide Tedion (R1, R2) or not selected (S; from Overmeer 1966). Hatching rates are high in the five crosses between lines of the same type, lower when R line females are crossed to ancestral S line males, and lowest when R line males are crossed to S line females ($p < 0.001$, analysis of variance comparing the three types of crosses). Among crosses of a given type, the variation in hatching rates only slightly exceeds that expected due to binomial sampling ($N = 162 - 657$ eggs per cross).

Recent studies on yeast (Dettman et al. 2007) and the filamentous fungus *Neurospora* (Dettman et al. 2008) give additional evidence that adaptation to different environments can generate postmating isolation. In the yeast study, replicate populations derived from a single diploid progenitor were allowed to adapt to either of two stressful environments, high salinity and low glucose, for five hundred generations. Diploid hybrids between populations from different environments showed substantially lower meiotic efficiency (percentage of cells undergoing meiosis under conditions that normally elicit sexual reproduction) than hybrids between populations from the same environment. Mitotic (as opposed to meiotic) reproduction of the hybrids on permissive medium was normal, however. In a study on *Neurospora,* Dettman et al. (2008) allowed replicate populations derived from either an interspecific cross or an intraspecific cross to adapt to either high salinity or low temperature. Two measures of postmating isolation were obtained: perithecial production (essentially fertilization success) in crosses between populations, and the percentage viability of spores resulting from successful crosses. For the experiments involving both progenitors, average spore viability under permissive conditions was lower in crosses between lines from different regimes than in crosses between lines from the same regime. The authors' statistical analysis, however, failed to distinguish replicate lines within regimes; thus, the true statistical significance of this and other results they obtained is difficult to evaluate. A clear result, however, was that perithecial production in populations derived from the interspecific crosses was dramatically depressed when females came from the high-temperature treatment and males came from the high-salt treatment; genetic analysis showed that this was likely the result of a two-gene interaction. Because these lines were derived from an interspecific cross, with resulting high linkage disequilibrium, there is a strong possibility that fixation of the incompatible allele combinations in the different regimes was driven by hitchhiking, rather than direct selection. Moreover, the authors did not rule out the possibility that the incompatibility simply recapitulated a difference between the parental species.

Clearly, more experiments are needed to determine how rapidly postmating isolation can evolve due to either selection or drift. Quantifying postmating isolation in its diverse forms, however, is more difficult than simply testing whether a pair of lines mate assortatively; this probably explains why postmating isolation has been relatively neglected in laboratory experiments on speciation.

How much gene flow is needed to prevent speciation by divergent selection? Although there is abundant evidence that divergent selection can lead to the evolution of reproductive isolation between allopatric populations, there is much less information on how readily reproductive isolation evolves between populations that are not completely separated by geography (Coyne and Orr 2004). An obvious experiment would be to subject laboratory populations to divergent selection with varying levels of gene flow between the selected extremes, choosing trait(s) for which divergent selection in allopatry is known to produce reproductive isolation. Surprisingly, this sort of experiment has rarely been done;

equally surprisingly, when it has been done, the results have suggested that substantial levels of gene flow need not greatly impede the evolution of reproductive isolation. Soans et al. (1974) and Hurd and Eisenberg (1975) subjected house fly populations to selection for positive and negative geotaxis with 0 percent (allopatric treatment; both studies), 30 percent (parapatric treatment; Soans et al.), and 50 percent (sympatric treatment; Hurd and Eisenberg) gene flow allowed between the selected extremes (note that the percentages refer to the potential gene flow that would occur in the absence of assortative mating and postmating isolation). In both studies, the treatments with gene flow were equally effective as the allopatric treatment in generating assortative mating. Grant and Mettler (1969) and Coyne and Grant (1972) selected *D. melanogaster* populations for high and low "escape" response (a measure that seems to combine negative geotaxis, positive phototaxis, and activity level) in allopatry and sympatry (Grant and Mettler) and later in parapatry (25 percent gene flow; Coyne and Grant). Highly significant premating isolation evolved in the allopatric treatment and in one of the two parapatric replicates, but not in the three sympatric replicates. While the results were not as striking as those of Hurd and Eisenberg (1975), the evolution of reproductive isolation in one of the two parapatric replicates in only ten generations suggests that lower but still substantial levels of gene flow (e.g., 5–10 percent) might not have impeded the evolution of reproductive isolation. More experiments of this type, with diverse organisms and traits, would help clarify the extent to which complete geographic separation is a prerequisite for the initial evolution of reproductive isolation.

How feasible are models of sympatric speciation via divergence in host or habitat choice? Specialization on different habitats or hosts can lead to premating reproductive isolation by causing populations to be physically separated at the time of mating. This has caused Bush (1975, 1994) and others (e.g., Via 2001) to champion the idea that host shifts can precipitate sympatric speciation in phytophagous insect species in which mating takes place on the host. Although there is evidence suggesting that host shifts have contributed to sympatric divergence in some groups (Via 2001) or at least help maintain divergence (Rundle and Nosil 2005), sympatric speciation is notoriously difficult to document in nature. Laboratory experiments can help clarify the feasibility of sympatric speciation via host or habitat shifts and identify the conditions under which it is most likely to occur.

Rice and Salt (1988, 1990) conducted an elegant test of one model of sympatric speciation via divergence in host or habitat preference, using *D. melanogaster*. The investigators built an elaborate maze that forced newly emerged flies to make three successive binary choices (light/dark, up/down, and odor 1/odor 2) before being able to find food and mate. This generated eight different artificial "hosts," each characterized by a unique set of stimuli. Only flies that chose two of the hosts, which required opposite sets of choices to locate (dark/up/odor 1 for host A, and light/down/odor 2 for host B), were allowed to contribute to the next generation, simulating the situation in which these were the only hosts suitable for development. Disruptive selection was simultaneously applied to development time, with only early-emerging flies that chose host A, and only late-emerging flies that chose host B, being allowed to breed. Within about thirty generations, gene flow between the two hosts ceased, because progeny of flies from host A were virtually never found in host B, and vice versa (Rice and Salt 1990).

Although Rice and Salt's experiment demonstrated that sufficiently strong disruptive selection on host or habitat choice can lead to sympatric divergence, a critical feature of the design was that only hosts requiring opposite sets of choices to locate were suitable for development. It is not clear how broadly applicable this scenario is to phytophagous insects and other host- and habitat-specific groups (to give a hypothetical example, if dark/wet and light/dry habitats are both suitable for development, why should dark/dry and light/wet habitats be lethal?). Moreover, the success of Rice and Salt's (1990) experiment seems to have been heavily dependent on the disruptive selection for development time, which can cause reproductive isolation only in species with nonoverlapping generations. From their figures 2–4, it does not appear that substantial divergence would have occurred based on selection for the opposite combinations of behavioral choices alone (with the possible exception of the treatment in which a somewhat artificial penalty of habitat switching was applied).

Interestingly, Bush's original verbal model of sympatric speciation in phytophagous insects (Bush 1975), which was partly inspired by the ideas of early entomologists, was based on very different assumptions from those simulated in Rice and Salt's experiment. Bush considered only two hosts, one ancestral and one novel. Mutations at separate loci arose which conferred acceptance of the new host, and ability to survive on it. A multilocus version of this model (Fry 2003) can indeed result in sympatric speciation, if alleles increasing viability on one host reduce viability on the other, and if there is sufficiently high genetic variance for viability and host preference, conferred by alleles with individually large effects.

A possible way to test the Bush model in the laboratory is diagrammed in figure 20.3. Emerging insects are allowed to choose between two hosts (real or artificial), after which mating takes place. Progeny of parents from the different hosts are kept separate and selected in the opposite direction for one or more quantitative traits, simulating the situation where the different hosts require opposite sets of traits for survival. After selection and emergence, insects from the two hosts are pooled and allowed to choose hosts again. This design differs from that of Rice and Salt (1990), because selection does not act directly on host choice (there are only two hosts, and insects are guaranteed to find one); instead, selection acts on the quantitative traits in a host-specific manner. This causes alleles affecting the selected traits to become associated with alleles affecting host preference (Diehl and Bush 1989; Fry 2003), resulting in indirect disruptive selection on host preference. In particular, even though genotypes with little or no host preference are no less successful at finding hosts than genotypes with strong host preference, they will tend to have intermediate values of the disruptively selected traits, and hence be maladapted to both hosts.

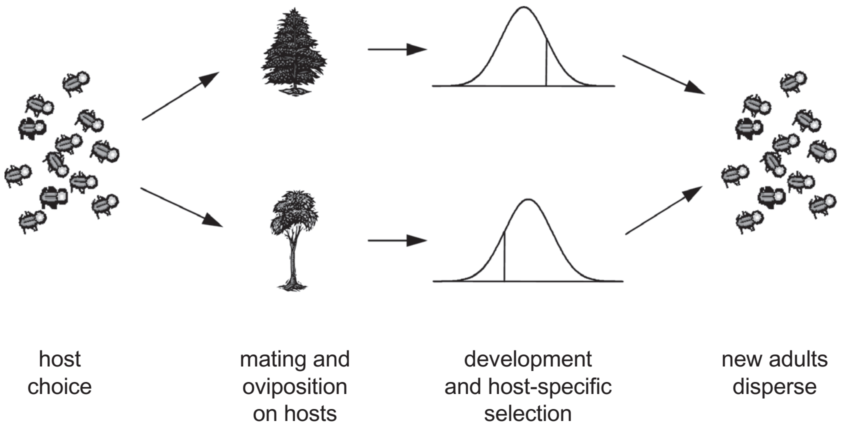

FIGURE 20.3
Hypothetical experiment for testing Bush's (1975) model of sympatric speciation. Nonmated insects are first allowed to choose between two real or artificial hosts; after host choice, the insects are allowed to mate and lay eggs. Depending on which host their parents chose, progeny are selected in opposite directions for one or more quantitative traits. The newly emerged adults are then allowed to choose hosts again, and the cycle repeated.

By using strong host-coupled disruptive selection on one or more highly heritable traits, the design in figure 20.3 mimics the situation where alleles increasing viability on one host reduce it on the other and where there is relatively high genetic variance for viability. Nonetheless, for population splitting ("speciation") to occur, there must be relatively high genetic variance for host preference as well. In a preliminary experiment on *D. melanogaster* in which flies were allowed to choose between two artificial hosts, however, genetic variance for host preference appeared to be too low to allow "speciation" (J.D.F., unpublished data). This does not necessarily sound the death knell for the Bush model of sympatric speciation, because *D. melanogaster* is not a host-specific phytophagous insect, and it may be a poor choice for such an experiment. A true phytophage population allowed to choose between two real hosts might have sufficiently high genetic variance for the scenario shown in figure 20.3 to result in population splitting. Indeed, crossing experiments on host races of *Rhagoletis* flies (Dambrowski et al. 2005) and *Nilaparvata* plant hoppers (Sezer and Butlin 1998) suggests that host preference in both groups is controlled by genes with large effects. Another possibility suggested by Fry's (2003) model, unfortunately not easily testable on the time scale of laboratory experiments, is that long-continued selection of the type shown in figure 20.3 would cause the genetic variance for preference to gradually increase, by favoring rare mutants with extremes of preference (see Fry 2003, figure 1) and/or suppressors of recombination between preference loci.

How readily does reinforcement occur? In the traditional reinforcement scenario, two populations diverge in allopatry and develop partial, but not complete, postmating reproductive isolation. When the populations again come into contact, selection favors mechanisms that reduce the likelihood of mating between them, because such matings produce low fitness hybrids. In the mid-1900s, reinforcement was widely regarded as a common, and perhaps even necessary, final step in speciation (reviewed in Coyne and Orr 2004). Although serious objections to the feasibility of reinforcement were raised in the 1980s (e.g., Butlin 1987), more recent theoretical models have suggested that reinforcement is plausible, and empirical evidence indicates that a process resembling reinforcement has occurred in some groups, most notably *Drosophila*. Nonetheless, the evidence for reinforcement in natural populations is subject to alternative explanations (Coyne and Orr 2004), and the appropriateness of the assumptions of the various proposed models of reinforcement remains to be verified.

Surprisingly, there have been few if any attempts to test realistic reinforcement scenarios in the laboratory. As noted earlier, reinforcement needs to be distinguished from reproductive character displacement, in which selection favors the avoidance of mating between species which already show complete postmating isolation; thus, the "destroy all the hybrids" experiments reviewed here test for reproductive character displacement, not reinforcement. In three of the destroy all the hybrids studies (Robertson 1966b; Harper and Lambert 1983; Hostert 1997; see table 20.1), the investigators also created treatments in which some hybrids were retained, and hence some gene flow between the hybridizing populations could occur. In the first two of these studies, however, no increase in premating isolation was observed even in the treatments without gene flow, indicating that the base populations were untypical in lacking the requisite genetic variation for mating-related traits. In Hostert's (1997) study, a small amount of premating reproductive isolation evolved in the zero gene flow treatment in twenty-five generations, but none was observed in three treatments with nonzero gene flow (theoretical gene flow of 3.3 percent, 10 percent, and 50 percent). While this result gives evidence against reinforcement, it is questionable whether Hostert's design mimics a realistic reinforcement scenario in nature. The parental populations were formed by backcrossing two recessive markers into the same genetic background and therefore should have been genetically and phenotypically similar, except for the effects of the markers themselves. In contrast, reinforcement is usually thought to apply to populations that have diverged in allopatry for a long period; such populations, even if incompletely reproductively isolated, would be likely to have multiple differences in morphology, mating behavior, and so forth, which could serve as the basis for reinforcement. Moreover, the use of recessive markers, which are likely to be associated with reduced fitness, may have resulted in the actual level of gene flow being higher than the theoretical levels, because the wild-type hybrids would probably have been more vigorous than the pure population parents.

A more biologically relevant way to test the feasibility of reinforcement would be to start with populations from different parts of a species' range and that already show considerable phenotypic divergence, if not partial reproductive isolation. (An alternative would be to start with laboratory populations that had previously been divergently selected for one or more quantitative traits). The simplest design would be an island-continent scenario, in which an experimental "island" population receives a small number of immigrants each generation from a nonevolving "continental" population (cf. the model of Kirkpatrick and Servedio 1999). If the two populations differ initially in (say) body size, then selection on body size could be used to create selection against immigrants. By varying the rate of immigration and the strength of selection on body size, one could determine whether there are conditions that permit some initial gene flow (i.e., hybrid fitness greater than zero) but that nonetheless result in the evolution of enhanced mating discrimination of island females against continental males. One of the challenges in such an experiment would be to carefully control the intensity of selection, to make sure one was not inadvertently performing a "destroy all the hybrids" experiment.

What is the role of sexual selection (including sexual conflict) in the evolution of reproductive isolation? The evolution of behavioral premating isolation is generally thought to require some form of sexual selection (Coyne and Orr 2004). The precise way in which sexual selection generates reproductive isolation is unclear, however. Verbal and theoretical models have suggested ways in which "good-genes," runaway, and sexual conflict processes, among others, could all result in premating isolation between allopatric populations (reviewed in Coyne and Orr 2004). Laboratory experiments represent a promising way to test the predictions of these models.

Recent experiments on sexual conflict are a promising start in this direction. According to the theory of sexual conflict (Holland and Rice 1998), the optimal number of matings is lower for females than for males; as a result, females are selected to resist mating attempts by males, and males are selected for the ability to overcome female resistance. This can result in perpetual antagonistic coevolution between males and females, possibly resulting in reproductive isolation between allopatric populations (i.e., because the males of one population may not have the requisite traits for overcoming the mating resistance of females of another population). An elegant experiment by Martin and Hosken (2003) on the dung fly *Sepsis cynipsea* provided support for this scenario. Females from populations kept under enforced monogamy, a regime that eliminates the potential for sexual conflict, showed relatively little reluctance to mate with males from either their own population or other monogamous populations after thirty-five generations. In contrast, females from populations kept in containers with multiple flies of both sexes, and hence with the potential for sexual conflict over mating rates, showed greater reluctance to mate in general, but especially with males from other replicate populations. Reproductive isolation was greatest between experimental populations kept at high density, and hence with high potential for sexual conflict, than between populations kept at a lower density, contrary to the prediction of drift-based models.

Although two recent experiments (Wigby and Chapman 2006; Bacigalupe et al. 2007) found no evidence of reproductive isolation between *Drosophila* populations maintained with the potential for sexual conflict, this is entirely unsurprising: the multiple previous speciation experiments with *Drosophila* used conditions that allowed sexual conflict but (as we have seen) rarely resulted in reproductive isolation evolving between lines maintained under the same conditions. It is thus likely that the Martin and Hosken's (2003) different results with dung flies stem from biological differences between *Sepsis* and *Drosophila* (Wigby and Chapman 2006). Experiments testing for the relationship between sexual conflict and reproductive isolation in other groups would help elucidate the importance of sexual conflict in speciation.

Sexual selection is also likely to play a role in the evolution of premating isolation between populations subject to divergent selection. If a selection regime were to change the mating preference of females for a particular male trait (see Rundle et al. 2005 for an example), this would automatically create directional sexual selection on the trait. Similarly, if a selection regime were to substantially change the mean of a sexually selected male trait, this would create selection for females to be willing to mate with males with previously unpreferred trait values. In fact, in the absence of sexual selection, independent responses to selection of female preference and the male traits that are the subject of the preference would be just as likely to result in disassortative mating (e.g., a preference of females from a given selection regime for males from a different regime) as in assortative mating. Nonetheless, the way in which sexual selection interacts with nonsexual selection to produce premating isolation among populations subject to divergent selection needs to be clarified. The simplest prediction is that divergent selection with enforced monogamy, and hence no opportunity for sexual selection, should result in less sexual isolation than divergent selection with the opportunity for mate choice.

What are the mechanisms by which reproductive isolation evolves? Most studies on the experimental evolution of reproductive isolation have given no information on the traits responsible for the observed reproductive isolation. Two studies on the evolution of cuticular hydrocarbons (CHCs), a type of pheromone, in *Drosophila serrata* populations provide promising, but incomplete, exceptions in this regard (Higgie et al. 2000; Rundle et al. 2005). Higgie et al. (2000) maintained *D. serrata* populations in bottles with and without a closely related species, *D. birchii*, whose natural range overlaps that of *D. serrata*. When the *D. serrata* base populations came from regions lacking *D. birchii,* the presence of the latter species caused their CHC profiles to evolve to resemble that of *D. serrata* populations from regions of overlap with *D. birchii*, suggesting reproductive character displacement. The authors did not, however, investigate the mating behavior of the evolved lines. Rundle et al. (2005) showed that maintaining *D. serrata* populations on different diets caused changes in female CHCs and, more surprisingly, female preference for particular CHCs, but they did not test whether premating isolation between lines on different diets evolved as a result.

A basic mechanistic distinction that needs to be addressed in further work is that between intrinsic and extrinsic isolation. Most studies have tested for only one type of isolation and thus give no information on their relative rates of evolution. Rice and Salt's (1990) study on sympatric speciation provides an interesting exception; although the experimental populations evolved extrinsic premating isolation due to differences in habitat selection, they showed no evidence of intrinsic premating isolation in mating tests. In their study of *Neurospora*, Dettman et al. (2008) measured progeny viability of interline crosses under permissive conditions and under the stressful conditions of the selection lines (high salinity and low temperature). While the results under permissive conditions gave some evidence for intrinsic postzygotic isolation, as noted earlier, viability of hybrids was particularly low when measured in the low-temperature environment (but not in the high-salinity environment), indicating that some extrinsic postzygotic isolation had also evolved. More studies comparing the speed of evolution and magnitudes of intrinsic and extrinsic isolation are needed.

GENERAL GUIDELINES FOR EXPERIMENTS ON SPECIATION

This review should make it clear that the literature on the experimental evolution of reproductive isolation has only scratched the surface of many important questions about speciation. Even those results that are relatively well replicated (e.g., that divergent selection often results in premating reproductive isolation, while population bottlenecks rarely do) come exclusively from experiments on flies. There is a clear need for innovative experiments on a broader range of species; recent studies on fungi (Greig et al. 2002; Leu and Murray 2006; Dettman et al. 2007, 2008) represent a promising start in this direction. Some questions (e.g., how often does postzygotic isolation evolve due to divergent selection?) could be addressed using selection lines created for other purposes, while others (e.g., how readily does reinforcement evolve?) will require experiments "from scratch."

To assist those designing new experiments, I offer a few general guidelines.

Carefully consider the base population The choice of base population is a crucial but often overlooked step in the design of any selection (or drift) experiment (see also Rhodes and Kawecki this volume; Rauser et al. this volume; Simões et al. this volume), experiments on speciation being no exception. Ideally, base populations should contain a broad sample of variation from a single, natural population. Starting a selection experiment from an inbred (whether deliberately or not) line will almost guarantee a weak response. At the other extreme, as pointed out by Rundle et al. (1998), the common practice of hybridizing lines from geographically diverse populations to establish the base population should also be avoided, because this may result in spurious outcomes with little relevance for natural populations. It may also be a good idea to allow the base population some time to adapt to laboratory conditions before the start of the experiment; otherwise, supposedly nonevolving "control" treatments may change rapidly due to laboratory adaptation (on the other hand, too long a period of laboratory adaptation may result in depletion of genetic variation; Templeton 1999). As noted earlier, experiments designed to test reinforcement-like scenarios arguably should start with populations that are differentiated to some extent.

Maximize effective population sizes and duration of experiments The response to selection is an increasing function of both the effective population size of the selected lines and the number of generations of selection (Falconer and Mackay 1996). Moreover, these factors interact, because small populations typically reach a selection limit sooner, as variation is depleted, than large populations (Weber and Diggins 1990). Because speciation is typically thought to require thousands of generations, selection experiments designed to test hypotheses of speciation should strive to maximize both effective population size and duration. This requires careful choice of both organism and experimental methods. Yeast and other sexual microorganisms have obvious advantages in this regard. Moreover, whenever possible, selection schemes should be devised which minimize or eliminate the need for manually scoring or measuring individuals, either by automating these steps or by performing "quasi–natural selection" experiments (aka "laboratory natural selection"—Rose and Garland this volume; Futuyma and Bennett this volume; Gibbs and Gefen this volume; Huey and Rosenzweig this volume), in which populations are simply allowed to adapt to different environments (e.g., different media or temperatures), obviating the need for phenotypic scoring and sorting.

Replication is crucial Two or more replicate lines per treatment should always be established (see also Rhodes and Kawecki this volume; Swallow et al. this volume). Different lines from the same treatment may behave very differently due to genetic drift. For example, Halliburton and Gall (1981) applied strong disruptive selection on pupal weight in the flour beetle, *Tribolium castaneum*; two replicate populations evolved strong reproductive isolation between the oppositely selected extremes, while the other two showed no evidence for reproductive isolation. Had the experiment not been replicated, the results might have tempted one to conclude that disruptive selection in this system is either likely or unlikely to result in reproductive isolation, depending on the result obtained. Moreover, in experiments to investigate whether divergent selection in allopatry produces reproductive isolation, a critical test is whether replicate lines from the same selection treatment show less reproductive isolation from each other than from lines from the opposite treatment. Without replicates, it would not be clear whether reproductive isolation observed between divergently selected lines resulted from the selection itself or from genetic drift (including "hitchhiking" of alleles causing reproductive isolation with those affecting the selected traits, which can be viewed as a form of drift in most instances).

In data presentation and statistical analysis, replicates must be clearly distinguished (see also Rhodes and Kawecki this volume). There is little point in creating replicate populations, only to later pool data from the different replicates for analysis (e.g., when conducting chi-square tests for nonrandom mating).

Know the relevant literature Experiments should be designed in a way that takes advantage of previous methodological advances. For example, anyone conducting tests for premating isolation should consult the literature on the most effective way to conduct and analyze mating trials (e.g., Spieth and Ringo 1983; Coyne et al. 2005).

Negative results should be reported For the literature to give an accurate picture of the efficacy of a given treatment (e.g., divergent selection) in producing reproductive isolation, and to avoid needless duplication of effort, negative results should be reported, even if this requires publishing in relatively "minor" journals or other outlets.

CONCLUSION

The last decade has seen a profusion of new approaches and ideas on speciation (reviewed in Barton 2001; Coyne and Orr 2004; Rundle and Nosil 2005; Noor and Feder 2006; Rieseberg and Willis 2007). A wealth of new theoretical models have been developed, genes contributing to reproductive isolation have been mapped and characterized, and new statistical methods for inferring the geographic mode of speciation have been applied. At the same time, experimental approaches have continued to give insights into mechanisms of speciation. While one should not lose sight of their limitations, particularly their short time scale and simplification of ecological conditions, laboratory experiments provide a powerful way to test the feasibility of theoretical models and to study the forces responsible for the initial evolution of reproductive isolation, something that is difficult to do with natural populations. They are therefore likely to continue to be an important part of the literature on speciation.

SUMMARY

Laboratory experiments on speciation investigate the conditions under which reproductive isolation can evolve between members of what was initially a single population, as well as the conditions under which reproductive isolation between initially partly reproductively isolated populations can become intensified. This chapter reviews past speciation experiments, emphasizing recent and previously overlooked studies, identifies neglected questions that could be addressed by new experiments, and gives guidelines for such experiments. In past experiments, partial premating (i.e., behavioral) reproductive isolation has sometimes evolved as a by-product of divergent selection on allopatric populations, but it has rarely evolved due to genetic drift alone. In contrast to the results of divergent selection in allopatry, application of strong disruptive selection to an initially random-mating population, a situation promoting sympatric speciation according to some models, has only rarely resulted in the evolution of premating isolation between the selected extremes. Nonetheless, there is some evidence that the different results of the allopatric and sympatric studies may have been partly caused by the different traits used for selection, not just the homogenizing effect of gene flow in the sympatric studies. Experiments in which disruptive selection is applied to a single trait known to be related to

mating behavior, with varying levels of gene flow between the selected extremes, could help clarify to what extent reproductive isolation can evolve in the face of ongoing gene flow. Other neglected subjects ripe for future experimental investigation include the roles of selection and drift in promoting postmating isolation, the feasibility of sympatric speciation via divergence in host or habitat preference, the conditions under which reinforcement can occur, and the role of sexual selection in the evolution of premating isolation. Speciation experiments have made important contributions to our understanding of mechanisms of speciation, and are likely to continue to do so, complementing comparative, genetic, and theoretical approaches.

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