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REPRODUCTIVE ISOLATION AND SPECIATION IN PLETHODONTID SALAMANDERS

STEVAN J. ARNOLD, NANCY L. REAGAN[1], AND PAUL A. VERRELL[2]

Department of Ecology and Evolution, The University of Chicago, 940 East 57th Street, Chicago, IL 60637, USA

THE aims of this paper are to outline briefly the current status of studies of reproductive isolation in plethodontids, to focus on the methods that have proved feasible in such studies, and to suggest some future directions for research. The rationales for particular questions about reproductive isolation are developed in the cited research papers and are only briefly mentioned in this review. It is not our intent to review past work and its motivations. Instead, we try to supplement the available literature by giving more detailed accounts of methods. Our comments about future directions are of course speculative and undoubtedly neglect some important possibilities. Current perspectives on reproductive isolation and speciation are reviewed in Futuyma and Mayer (1980), Giddings et al. (1989), and Otte and Endler (1989).

Plethodontid salamanders offer an exceptional opportunity to advance our understanding of speciation and the evolution of reproductive isolation. The geographic distribution and habitat requirements of most species are well understood after several generations of extensive fieldwork (e.g., Dunn, 1926; Hairston, 1949, 1973; Hairston et al., 1992; Organ, 1961; Tilley, 1973; Wake, 1987; Wake and Lynch, 1976). Phylogenetic relationships within many of the family's tribes have been elucidated by a series of careful systematic studies (reviewed by Highton, 1991; Larson and Chippendale, 1993; Wake, 1993). Courtship behavior is evolutionarily conservative within the family and well understood (Arnold, 1977; Houck and Verrell, 1993). The salamanders themselves are often abundant in nature, easily collected, and readily maintained in captivity in large numbers. These attributes mean that experimental studies of reproductive isolation are feasible. Among vertebrates, only anurans and fishes offer comparable opportunities for experimental studies of speciation, although few species in these two groups provide opportunities for staging large numbers of actual mating trials.

The most illuminating material for study are populations in the process of speciating or species in statu nascendi, to use Dobzhansky's (1937) memorable phrase. In other words, we need to identify pairs of sister taxa poised at crucial stages of divergence in traits that affect reproductive isolation. Until the advent of modern molecular methods, the identification of actively speciating populations was often based solely on qualitative assessment of divergence and morphological diagnosis of contact zones (Mayr, 1942, 1963). Molecular methods enable the investigator to assess accurately the degree of gene flow between populations (Larson et al., 1984; Slatkin, 1985, 1987). Furthermore, molecular techniques (particularly mtDNA analysis) can elucidate infraspecific phylogenies (Avise, 1989; Avise et al., 1987). By combining molecular systematics with experimental studies of reproductive isolation, one can focus on sister taxa in the process of speciating.

Two kinds of data from studies of systematics set the stage for experimental studies of reproductive isolation. First, we can detect zones of hybridization or introgression using allozymes or other genetic markers (e.g., Duncan and Highton,

[1] PRESENT ADDRESS: Department of Entomology, University of Kentucky, Lexington, KY 40546-0091, USA.

[2] PRESENT ADDRESS: Department of Zoology, Washington State University, Pullman, WA 99164, USA.

1979; Highton and Henry, 1970; Peabody, 1978; Wake et al., 1980; Wake and Yanev, 1986). Second, we can detect genetic differentiation across broadly distributed contiguous or allopatric arrays of populations using genetic markers (e.g., Highton and Webster, 1976; Larson and Highton, 1978; Tilley et al., 1978). Thus, Reagan (1992) based her studies of sexual isolation in the *Plethodon jordani-glutinosus* complex on the allozyme and morphological diagnoses of contact zones by Highton and Henry (1970) and Peabody (1978). Verrell and his colleagues (Tilley et al., 1990; Verrell and Arnold, 1989; Verrell et al., 1993) based their studies of sexual isolation among allopatric populations of *Desmognathus ochrophaeus* on the allozyme survey of Tilley et al. (1978).

Experimental Studies of Sexual Isolation

Sexual isolation is currently the most researched component of reproductive isolation in plethodontids. Because such work provides a foundation upon which other studies can be built, we will first briefly review previous results. We will then discuss criteria and protocols that have proven useful in sexual isolation studies.

The basic procedure in an experimental study of sexual isolation is to stage sexual encounters between animals from different populations and score their outcome. The goal is to determine what happens when sexual partners meet one another. The rationale for staging such encounters in the laboratory is that (1) the behavioral events that happen after encounter are likely to be the same in the laboratory as in the field (Houck and Verrell, 1993) and (2) replication is far easier in the laboratory. Sexual isolation is a part of the total reproductive isolation that may thwart gene flow between two populations (Dobzhansky, 1937; Mayr, 1963). In contrast, the study of ecological isolation addresses the issue of whether potential sexual partners *do* encounter one another. The study of post-mating isolation is concerned with events that happen after insemination. Thus, no study of sexual isolation can claim that all of reproductive isolation has been analyzed; rather, one component of isolation is measured and dissected in detail.

Studies of allopatric, but putatively conspecific, populations of several species have revealed levels of sexual isolation ranging from nonexistant (random mating) to complete (*Plethodon jordani*: Reagan, 1992; *Desmognathus santeetlah*: Maksymovitch and Verrell, 1993; *D. ochrophaeus*: Houck et al., 1988; Tilley et al., 1990; *D. imitator*: Verrell and Tilley, 1992). The strength of sexual isolation either showed no relationship with genetic or geographic distance among populations, as in *P. jordani* (Reagan, 1992), or was best predicted by geographic distance, as in *D. ochrophaeus* (Tilley et al., 1990). Clearly, divergence of mate-recognition systems may arise among geographically isolated, conspecific populations.

Significant levels of interspecific sexual isolation have been detected among both allopatric and sympatric populations of *Desmognathus* (Houck et al., 1988; Verrell, 1990*a,b,c*; Verrell and Tilley, 1992; Uzendoski and Verrell, 1993). Reagan (1992) found that sexual isolation was statistically stronger between populations of *Plethodon jordani* and *P. teyahalee* in zones of sympatry with no apparent hybridization than between allopatric populations. This result is consistent with models of speciation in which selection reinforces sexual isolation between populations upon secondary contact. Sexual isolation was not stronger between the two species from an area where they form a narrow hybrid zone than in allopatry (Reagan, 1992). The strength of sexual isolation between *P. jordani* and *P. teyahalee* from two areas where they naturally hybridize was similar; these results suggest that factors other than sexual incompatibility control the width of these hybrid zones (Reagan, 1992).

Behavioral observations have revealed that heterotypic courtships tend to be less frequent than homotypic courtships, and that when the former are initiated, they often break-down prior to tail-straddle-walk, which signals both partners' interest in and commitment to mating (Reagan, 1992; Verrell and Arnold, 1989). In some

heterotypic crosses, courtships are not even initiated, either within species (Verrell and Arnold, 1989) or between species (Arnold, 1976; Uzendoski and Verrell, 1993; Verrell, 1989*a*). These findings strongly support the view that chemical cues play an important role in sexual isolation in plethodontids (Arnold, 1976; Dawley, 1984, 1986*a*,*b*, 1987; Noble and Brady, 1930). It seems that female cues are vital stimuli in releasing initial courtship interest from males (Uzendoski and Verrell, 1993).

Attributes of Good Subjects

Sexual isolation is vastly easier to study in some plethodontid species and genera than in others. The main attributes that promote feasibility are (1) ease of field collection, (2) tendency to court in the laboratory, and (3) length of courtship season. For example, most species of *Desmognathus* are more accessible to field collection than *Gyrinophilus*. A study of sexual isolation in *Gyrinophilus* might be restricted to a single pair of localities or to small samples from many sites. A study using *Desmognathus*, *Eurycea*, or *Plethodon* would not be so constrained. Some taxa court readily in the laboratory (e.g., *Desmognathus*, *Eurycea*, large eastern *Plethodon*), but others do not (e.g., *Ensatina*, *Gyrinophilus*, *Plethodon cinereus*). Even within the first group, rates of spermatophore deposition per evening for pairs from the same population range from about 60% in *Desmognathus ochrophaeus* to about 30% in *Plethodon jordani* and *P. teyahalee* (Reagan, 1992; Verrell et al., 1993).

While the physiological basis of these differences is not understood, the consequence is that orders of magnitude more data can be gathered using some species rather than others. The other main factor affecting the rate of data collection is the length of courtship season. At one extreme are genera with seasons of courtship (or accessibility to collection) that last only a few weeks (e.g., *Batrachoseps*), and at the other extreme are genera with seasons that last a few or several months (e.g., some *Bolitoglossa*, *Desmognathus*, large eastern *Plethodon*). A pilot study that assesses the proportion of pairs from the same population that will court in a single night (and the seasonality of that statistic), for several populations, is an important prelude to a study of sexual isolation.

Hormone therapy is not a ready solution for species that are not inclined to court in captivity. The problem is that we do not know how LH-RH and other hormones affect the details of courtship within populations, much less the outcome of between-population trials.

Techniques for Successful Long-term Maintenance of Animals

A successful program of experimental research with plethodontids, or any organism, depends on good techniques for long-term maintenance. Animals must be in absolutely peak condition in order to court and successfully complete the other components of reproduction. We maintain plethodontids individually to eliminate fighting and resource competition and to prevent unscheduled sexual encounters. Clear plastic boxes make excellent cages for such individual maintenance and are available in a variety of sizes [e.g., shoe box size (29 × 15 × 8.5 cm) for large animals, small box size (17 × 12 × 6 cm) for small animals]. For a substrate, we use damp, brown paper towels or live *Sphagnum* moss; a crumpled damp towel is also provided as a refuge. Maintenance boxes may be checked weekly for moisture, but they only need to be cleaned on a monthly or bimonthly schedule; indeed, the less disturbance the better.

We use *Drosophila* as a routine food for small plethodontids (e.g., *Batrachoseps*, *Bolitoglossa*, *Desmognathus*, *Eurycea*, small eastern *Plethodon*), keeping active culture vials in the maintenance box at all times. Such vials can often be obtained from geneticists (by interception before vial washing) and provide a continually renewing source of larvae, pupae, and adults. *Drosophila* may not be a nutritionally complete diet, so we offer each salamander one or two waxworm moth larvae (*Galleria mellonella*), dusted with multiple vitamin and mineral powder, every week or two. These larvae may be obtained from dealers or cultured. We feed larger pleth-

odontids (*Ensatina, Gyrinophilus,* large eastern *Plethodon, Pseudotriton*) dusted waxworms and crickets (*Achaeta domestica*). *Tubifex* worms are excellent food for aquatic larvae.

Overheating (body temperatures in excess of 20 C for temperate or high elevation forms or in excess of about 25 C for lowland bolitoglossines) is injurious to plethodontids. We keep our maintenance and experimental rooms at 15–17 C. Rooms are kept under a natural photoperiod controlled by an outside photocell. Many plethodontids (especially *Ensatina, Eurycea,* and *Desmognathus*) will thrive for years under the conditions that we have outlined.

Scoring the Outcome of Sexual Encounters

The outcome of sexual encounters can be scored even when sexual behavior has not been observed. We stage encounters by placing a single pair (one adult male and one ovigerous female) in a plastic box just before dark. Pairs are left together overnight under dim red illumination. The outcome can be scored the next morning by examining the female and the box substrate (a damp paper towel pressed flat against the floor of the box). Insemination can be scored by pressing the female against the transparent side of the box and examining her cloaca for a white sperm mass (Fig. 1). Spermatophore deposition can be scored (whether or not insemination occurs) by carefully checking the substrate for spermatophore bases (Fig. 2; Arnold, 1976; Organ and Lowenthal, 1963). Such data can be readily converted to the spermatophore, transfer, and insemination scores described by Houck et al. (1985*a*). These scores measure, respectively, the probability that one or more spermatophores have been deposited, the probability of insemination given the deposition of one or more spermatophores, and the overall probability of insemination.

Experimental Protocols for Measuring Sexual Isolation

The goal in a study of sexual isolation between two populations is to estimate the frequency of mating success in four types of encounters (Table 1). A good estimate of each encounter type will be based on many trials (say, 20 or more of each type). We use a single pair of animals (one male and one female) in each trial to avoid complications due to male-male fighting and other forms of sexual interference. If we use different pairs of animals in every trial, statistical analysis of the data will be easy, but we will need a large number of animals. Alternatively, we can use the same animals in more than one trial, allowing a

FIG. 1.—Ventral view of a recently inseminated, ovigerous female *Desmognathus ochrophaeus*. A whitish sperm mass is visible in her cloaca. Photograph by L. Houck.

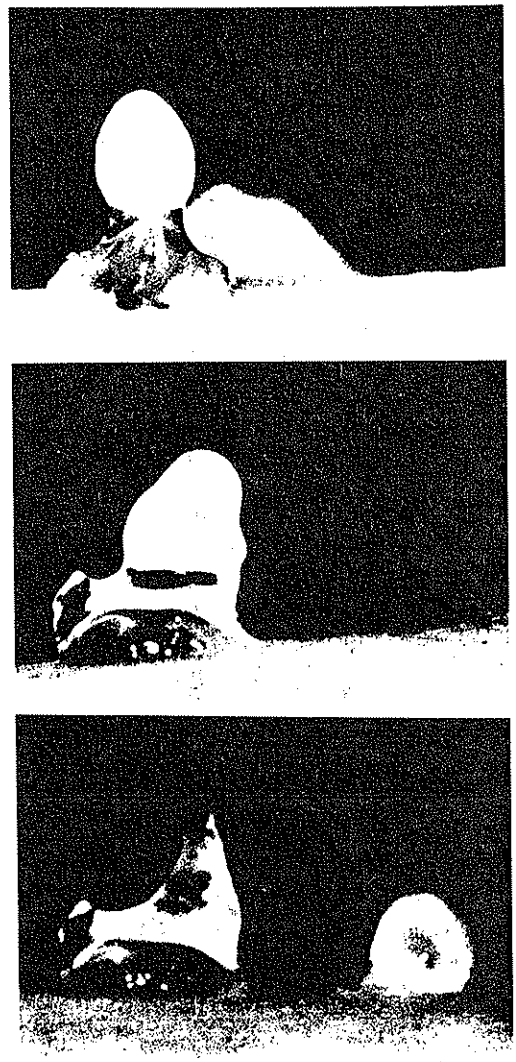

FIG. 2.—The spermatophore of a *Plethodon jordani* from Tusquitee Bald, Clay/Macon Co., North Carolina. **Top.**—Posterior view of the intact spermatophore. **Middle.**—View of the right side of the intact spermatophore (height is about 3.8 mm). The spermatophore was deposited by a male facing to the right. **Bottom.**—View of the right side of the spermatophore base (left) and a ventral view of the sperm mass (right), showing the cavity into which the spermatophore base inserts. Photographs by J. Organ.

TABLE 1.—Results of a test for sexual isolation between two populations of *Desmognathus ochrophaeus*. Population A is Rough Butt Bald and population B is Whiteside Mountain, two localities 31 km apart in North Carolina (see Tilley et al., 1978, for locality details). **Above.**—Summary of insemination results. Thirty trials of each of the four types of matings were accomplished in the experiment. The number in the numerator in each cell is the number of trials resulting in insemination. Standard errors are given after ±. **Below.**—Calculation of the three measures of sexual isolation described in the text.

	Male population	
	A	B
	21/30	20/30
	A = 0.70 ± 0.12	= 0.67 ± 0.12
Female population		
	7/30	20/30
	B = 0.23 ± 0.10	= 0.67 ± 0.12

Joint isolation = (0.70 + 0.67) − (0.67 ± 0.23) = 0.47 ± 0.08
Isolation asymmetry = |0.67 − 0.23| = 0.44 ± 0.16
Propensity asymmetry = |0.70 − 0.67| = 0.03 ± 0.17

period of recovery between successive trials of at least four nights to ensure that animals remain sexually motivated (Verrell, 1988*a*, 1991).

To use multiple trials with each animal, however, one must carefully balance the scheduling of trials or the resulting data may not be interpretable statistically. Our experimental designs are based on the following considerations. The females and males in each population will each encounter two types of mating partners. If each individual encounters both types of partners, the total number of encounters for each animal must be an even number. The overall tendency to mate may change over the course of the experiment, so we do not want to group all encounters of one kind at the start or end of the trial series. A ready solution is to alternate the encounter types for each animal. Consider the first trial for females from one of the populations. Which type of mating partner should they encounter? The best answer is "both types", but to accomplish that we will need to partition the females into two teams. One team starts with one type of partner, the other team starts with the other type. Following this logic, the males also need to be split into two teams. In statistical jargon, we have counterbalanced the trial sequences for both sexes and populations; consequently, any sequence effects should cancel out in data analysis. We also need to decide how many

TABLE 2.—Assignment scheme for mating partners used to produce the sexual isolation results reported in Table 1. The two teams of females from population A are denoted A and A'. Likewise, there are two teams each for population B females, population A males and population B males. For example, on the first trial night (4 June) female 1 on team A was paired with male 1 on team A and on the second trial night (8 June) she was paired with male 4 on team B.

	Trial night					
	4 June	8 June	12 June	16 June	20 June	24 June
Females	Males					
A team 1	A team 1	B team 4	A team 5	B team 1	A team 4	B team 5
2	5	5	3	3	1	2
3	2	1	1	4	3	3
4	4	2	2	5	5	4
5	3	3	4	2	2	1
A' team 6	B' team 9	A' team 9	B' team 10	A' team 7	B' team 6	A' team 8
7	8	8	9	9	7	6
8	6	6	7	10	10	9
9	10	7	8	6	9	10
10	7	10	6	8	8	7
B team 1	A' team 9	B' team 9	A' team 7	B' team 10	A' team 10	B' team 8
2	7	6	9	7	6	10
3	8	7	6	9	7	6
4	10	10	8	8	9	9
5	6	8	10	6	8	7
B' team 6	B team 5	A team 2	B team 3	A team 4	B team 2	A team 1
7	4	1	5	2	1	5
8	1	4	2	5	5	3
9	3	3	1	1	4	4
10	2	5	4	3	3	2

mating partners of each type will be encountered. A simple solution is to have each animal encounter every possible partner on both teams. Such a complete pairing scheme can result in a lengthy experiment. For example, if we stage trials every fourth night and there are five animals on each team, the experiment will last 40 nights. We often use an incomplete pairing scheme in which each female encounters a random subset of males from each team. For example, in the design shown in Table 2, each female encounters only three of the five males on each of two teams.

Measures of sexual isolation are based on the frequency of mating success in four types of encounters (Table 1). No one measure captures all the components that contribute to isolation, and so a variety of measures have been proposed (Bateman, 1949; Gilbert and Starmer, 1986; Levene, 1949; Levene and Dobzhansky, 1945; Malagolowkin-Cohen et al., 1965; Merrell, 1950). If we accept the need for multiple measures, a good argument can be made for making each as simple as possible. If we make our measures complicated functions of the four mating frequencies, they will have the undesirable property of large standard errors. Measures that are standardized to range from minus one to plus one (e.g., measures proposed by the above cited authors) suffer from this problem. Instead, we have used three unstandardized measures to quantify components of sexual isolation (Reagan, 1992; Tilley et al., 1990; Verrell et al., 1993). *Joint isolation* (the sum of the proportion of successful homotypic encounters minus the sum of the proportion of successful heterotypic encounters) measures the overall tendency for heterotypic encounters to be unsuccessful. *Isolation asymmetry* (the absolute difference between the two proportions of matings in the two types of heterotypic encounters) measures discrepancy in the success of the two heterotypic encounters. *Propensity asymmetry* (the absolute difference between the two proportions of matings in the two types of homotypic en-

counters) measures discrepancy between the two source populations in mating tendency. Each of these measures can be calculated with frequencies of spermatophore transfer or insemination success. We have usually assessed sexual isolation using frequencies of insemination success (Table 1).

Standard errors for measures of sexual isolation are needed for hypothesis testing. For example, does a measure of isolation truly differ from zero (indicating statistical significance), or do two pairs of populations differ in degree of isolation? In our experimental design, formulae for standard errors are complicated, because multiple trials with the same animal cause the trials to be nonindependent. The covariance between trial outcomes from this source must then be figured into the standard error formulae. The computation of standard errors of the four mating frequencies in the experimental design that we have outlined (Table 2) is derived and explained by McCullagh and Nelder (1989). In a later paper, we intend to show how those results can be extended to other mating designs.

Identifying the Determinants of Sexual Isolation

The protocol outlined above can inform us whether or not two populations engage in courtship interactions that culminate in spermatophore deposition or insemination. But it cannot discriminate between cases where no heterotypic courtship occurs from those in which courtship fails to progress as far as spermatophore deposition. In addition, it cannot identify the cause of sexual isolation. We have found it useful to work toward the identification of causes in a series of steps (which also may be executed simultaneously).

(1) Our first step is to survey a series of allopatric populations or allopatric/sympatric species, testing for sexual isolation in as many pairs as possible (Maksymovitch and Verrell, 1993; Reagan, 1992; Tilley et al., 1990; Uzendoski and Verrell, 1993; Verrell, 1990*a*,*b*,*c*; Verrell and Tilley, 1992). Such a survey can tell us which pairs of populations show the most extreme isolation. These are the pairs of populations where detailed behavioral observation is most likely to be informative.

(2) Our next step is to observe systematically the behavioral interactions in homotypic and heterotypic encounters (Reagan, 1992; Verrell, 1989*a*, 1990*b*; Verrell and Arnold, 1989). At this step, we use the same mating design as in step 1 (Table 2), but on each trial night we observe courtship progress in all the pairs of animals. Continual observation is desirable but impractical, especially when large numbers are under study. Instead, we arrange the clear plastic boxes used in mating trials in stacks and scan the stacks for courtship at preset intervals (Altmann, 1974), usually once every 20 or 30 min. Because courtship in plethodontids is a relatively leisurely affair, we do not miss major behavioral transitions with this frequency of sampling. Typically we score interactions at each time-point on a qualitative scale of increasing courtship progression (ranging from the lowest score of "no interest, animals apart" to the highest score of "spermatophore deposition and transfer". Although somewhat coarse-grained, the results of such scan-sampling enable us to identify the courtship stage(s) where progress is prone to break off (e.g., Houck and Reagan, 1990; Verrell and Arnold, 1989).

For those heterotypic encounters of most interest, we often use videotaping as a follow-up to scan sampling in order to obtain fine-scale data (Maksymovitch and Verrell, 1992; Uzendoski and Verrell, 1993). Our videosystem (Panasonic) works under very low-level illumination, and we usually record using time-lapse (increasing tape-time 12-fold). Where we do not require too much detail on a per-encounter basis, we use a switching device connected to multiple cameras in order to sample as many as four encounters in rapid succession (usually at intervals of <30 s) on a single videocassette recorder.

(3) Our third step is truly manipulative, and is best illustrated with an example. In encounters between *D. ochrophaeus* and *D. fuscus*, and between *D. ochrophaeus* and *D. imitator*, observations indicate that males do not even initiate the most prelim-

inary of courtship interactions with heterospecific females (Uzendoski and Verrell, 1993; Verrell, 1989*a*); it is as if males fail to recognize such females as potential mates. Using a technique pioneered for plethodontids by Dawley (1984, 1986*a*,*b*, 1987), we have presented males with substrate-borne female chemical cues in the arms of Y-shaped mazes, or olfactometers. Males demonstrate significant preferences for entering arms containing homospecific female cues, a result consistent with failure to recognize or repulsion by cues from heterotypic females.

Future Directions

Sexual isolation is only one component of a series of incompatibilities (both pre- and post-mating) that can result in reproductive isolation among taxa. There is a clear need for additional studies of sexual isolation in plethodontids, especially for species occupying broad geographic ranges. In addition, we identify three further areas for research: (1) other components of reproductive isolation, (2) the behavioral basis of sexual isolation, and (3) interactions in contact zones.

Ecological Isolation

The extent to which subtle differences in habitat preferences could produce sexual isolation (Dobzhansky, 1937) has not been addressed for plethodontid salamanders. Indeed, rates of insemination seem to be relatively unaffected by the "habitat complexity" of laboratory courtship arenas, at least for homotypic encounters (Verrell, 1988*b*). Nevertheless, such differences could plausibly promote reproductive isolation. For example, *D. ochrophaeus* from a stream-side population might preferentially use a horizontal surface, whereas those from a rock-face might use vertical surfaces. We were unable to find any obvious differences in the extent of sexual isolation among populations when we partitioned our data-set to compare pairs of populations from similar and dissimilar microhabitats. A better approach, modelled on Wecker's (1963) study of *Peromyscus*, might be to examine sexual isolation among populations held in field enclosures in different habitats, i.e., A and B in A's habitat versus A and B in B's habitat (including all homotypic controls). The need for studies of ecological isolation is most urgent for sympatric populations, for which we advocate detailed field studies of differences in habitat preferences and utilization. Results from these may be used to determine likely rates of heterotypic encounter.

Temporal Isolation

Population differences in the timing of courtship seasons can produce temporal isolation (Dobzhansky, 1937). Protocols already described can be used to address this issue. For example, our survey of *D. ochrophaeus* suggests that there are only minor differences among populations in courtship season, as measured by homotypic mating propensity (Verrell et al., 1993). Nevertheless, we suggest that repeated sampling from nature through seasons is preferable to long-term maintenance with periodic staging of courtship trials. Such sampling might portray field differences in courtship seasonality more accurately than comparisons made with long-term captives. Once again, temporal isolation is of special significance for sympatric populations. Potential rates of heterotypic encounter could be estimated from the results of detailed field studies of differences in activity patterns.

Mechanical Isolation

Populations that are physically unable to effect sperm transfer, despite engagement in courtship, are said to show mechanical isolation (Dobzhansky, 1937). Sperm transfer is indirect by means of a spermatophore deposited on the substrate in all plethodontids. Given the choreography of tail-straddle walk, the stage of courtship when sperm transfer occurs (Arnold, 1977), population differences in body size might be expected to influence insemination success. However, we have been unable to detect any contribution of size differences to sexual isolation among populations of *D. ochrophaeus*, or between *P. jordani* and *P. teyahalee*. It is also possible that differences in spermatophore struc-

ture might influence insemination success. However, spermatophores appear to be evolutionarily conservative within the family, and no obvious structural differences have been detected among conspecific populations of *P. jordani* and *D. ochrophaeus*.

Determinants of Sexual Isolation

Previous work indicates that chemical cues play an important role in the maintenance of sexual isolation. Plethodontid courtship is characterized by diversity in behavior patterns used to transfer courtship pheromones from males to females (Arnold, 1977; Houck and Arnold, 1982). Within a population, these pheromones increase the rate at which females become fully sexually responsive (Houck and Reagan, 1990). However, it is unknown whether differences in transfer behavior and/or pheromone chemistry might be responsible for sexual isolation among populations and species. Our work on *D. ochrophaeus* suggests that, for at least some pairs of populations, heterotypic encounters are prone to break down at or shortly after the time when courtship pheromones are transferred (Verrell and Arnold, 1989). Ultimately, the demonstration of differences in courtship pheromones rests with biochemical characterization. However, behavioral experiments may prove useful even in the absence of such data. The experimental protocol developed by Houck and Reagan (1990), in which females exposed to extracts of male courtship glands are paired with surgically-glandless males, could easily be modified to address sexual isolation, through separation of male behavior from courtship pheromone chemistry. For example, females of A could be exposes to glandless males of A (homotypic behavior) but gland extracts of B males (heterotypic pheromones).

Post-zygotic Isolation

Experimental studies of post-zygotic isolation have not yet been accomplished in plethodontids, although most of the requisite design elements are feasible. Post-zygotic isolation refers to a whole series of genetic and developmental incompatibilities that are expressed in and after the F_1 zygote stage, and includes inviability, sterility, or fecundity impairment in one or both sexes (Dobzhansky, 1937; Mayr, 1963). Post-zygotic isolation is an important precondition for reinforcement of pre-zygotic isolation in sympatry (Mecham, 1961). We would also like to know whether post-zygotic isolation evolves more slowly than pre-zygotic isolation (Coyne, 1992; Coyne and Orr, 1989). The individual husbandry steps that are necessary to study post-zygotic isolation have been accomplished in our own and in other laboratories. The challenge is to execute those steps in sequence and with the replication required in an experimental protocol.

To study post-zygotic isolation, one would need to rear plethodontids through at least one complete life cycle in the laboratory. A convenient starting point is sexually immature individuals from two or more natural populations. Collection as juveniles is a necessary step in obtaining females that have never mated and so have no stored sperm. Mated females may store viable sperm across successive breeding periods, thus rendering the assignment of paternity impossible (Houck and Schwenk, 1984). We have reared over 1000 individual *D. ochrophaeus* to sexual maturity in 6–18 mo in our laboratory (the time taken being dependent on age and size at capture). The next step is to stage matings within and among populations (using protocols already described), and then obtain fertilized eggs. Ovulation and oviposition rarely occur spontaneously in the laboratory, but can be induced in plethodontids with the protocol of endocrine manipulation using LH-RH described by Verrell (1989*b*). This protocol works very well in inducing field-caught females to lay, but has so far proved disappointing with laboratory-reared females.

Once eggs have been deposited, we have found it convenient to remove them and rear them individually or in groups under standardized conditions in perforated plastic Tissue Tek capsules (TM-Miles Laboratories, Inc.). These capsules are placed in plastic shoe boxes floored with damp paper towels. Each morning, we carefully dunk each capsule into deionized tap water and then carefully place each egg into

its own depression in the capsule's grid-like floor. Although time consuming, this protocol results in a rate of hatching close to 100% for *D. ochrophaeus* (Houck et al., 1985*b*).

Once a successful rearing protocol has been established, the next phase is to score the development, viability, sterility, and fertility of individuals produced from hybrid and control crosses. An obvious first step is to score the proportions of zygotes that develop to various landmark stages (e.g., gastrulation, neurula, tail bud). A surprising shortcoming of the plethodontid literature frustrates even this first step. We can find no detailed, published developmental tables for any plethodontid species! Thus progress in experimental studies of developmental incompatibility depends on further progress in the description of plethodontid embryology. A complete study of post-zygotic isolation will require that surviving F_1 individuals be raised to sexual maturity in order to test for sterility or dysgenesis in F_2 individuals.

Interactions in Zones of Contact

Hybridization between parapatric or sympatric species is relatively uncommon in plethodontids, suggesting that most populations became well-differentiated prior to secondary contact. Evidence for hybridization is available both within species (Guttman and Karlin, 1986; Hairston et al., 1992; Highton, 1970, 1977; Wake and Yanev, 1986; Wake et al., 1986, 1989; Yanev, 1978) and between species (Duncan and Highton, 1979; Highton, 1970, 1983; Highton and Henry, 1970; Karlin and Guttman, 1981; Peabody, 1978; Tilley, 1981, 1988; Wake et al., 1980; Wake and Lynch, 1982). The zones of contact identified in these studies provide outstanding opportunities to study the role of natural selection in the evolution of sexual isolation, the dynamics of hybridization, and its consequences.

Selection for sexual isolation.—Whether or not selection can produce or reinforce sexual isolation between species in secondary contact is a question of intense debate (Otte and Endler, 1989). The usual experimental approach is to compare the strength of isolation between sympatric and allopatric populations of two species. Populations are chosen on the basis of geographic distribution, with no explicit regard to phylogenetic relationships. Such choice poses no problem when populations are contiguous over relatively small areas, such as altitudinal contact zones between *P. jordani* and *P. teyahalee* (Hairston et al., 1992; Reagan, 1992). However, we advocate a phylogenetic approach to choice when populations are geographically disjunct over wide areas: e.g., *D. ochrophaeus* and *D. imitator* (Tilley et al., 1978; Verrell and Tilley, 1992). In this latter case, appropriate allopatric populations should be sister taxa to those in sympatry.

A second goal for future studies is to couple studies of sexual isolation with studies of post-mating isolation, such as hybrid dysgenesis. Such a dual approach is vital if we are to address the role of selection against hybrids in driving the evolution of sexual isolation in areas of secondary contact.

Hybrid zones.—As Harrison (1990) stated, hybrid zones are "windows" on important evolutionary processes. The origin and temporal stability of such zones are issues of considerable interest (Hairston et al., 1992). Studies of sexual isolation, fertilization success, and hybrid fitness, combined with molecular studies of gene introgression, can be used to evaluate the stability of hybrid zones. For zones presumed to be stable, such data can be used to determine whether selection against hybrids and migration of parentals are balanced, or whether hybrids are favored over parentals by selection for local adaptation (e.g., Arntzen and Wallis, 1991; Sanderson et al., 1992; Szymura and Barton, 1991). A caveat is that *in*stability can only be detected with long-term studies (Hairston et al., 1992).

In addition, our ability to reconstruct phylogenies and our concept of species are challenged by the existence of hybridization and "reticulate" speciation (Arnold, 1992; Grant and Grant, 1992; McDade, 1992). The magnitude of these challenges depends on the stability of hybrid zones (see above) and the role of hybridization in speciation. That plethodontid salamanders may prove model systems for ad-

dressing the latter issue is indicated by the fact that some taxa may be hybridogenic in origin: e.g., *P. teyahalee*, a hybrid between *P. jordani* and *P. cylindraceus* (Highton et al., 1989).

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