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Paul A. Verrell; Stevan J. Arnold

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BEHAVIORAL OBSERVATIONS OF SEXUAL ISOLATION AMONG ALLOPATRIC POPULATIONS OF THE MOUNTAIN DUSKY SALAMANDER, *DESMOGNATHUS OCHROPHAEUS*

PAUL A. VERRELL AND STEVAN J. ARNOLD
Department of Ecology and Evolution, The University of Chicago, 940 East 57th Street, Chicago, IL 60637

Abstract.—The behavioral basis of sexual isolation was investigated in 11 crosses staged between individuals from nine allopatric populations of the Appalachian mountain dusky salamander, *Desmognathus ochrophaeus*. Scan-sampled observations of within-population (homotypic) and between-population (heterotypic) heterosexual encounters yielded the following results. 1) Fewer heterotypic encounters than homotypic encounters resulted in insemination of females. Indices of the strength of sexual isolation based on insemination data ranged from 0.26 (lowest) to 0.82 (highest), indicating that mechanisms that confer sexual isolation may evolve during allopatric differentiation. 2) Heterotypic encounters that progressed as far as the stage of courtship during which the male stimulates the female to mate did so at the same time as homotypic encounters, although the number of the former reaching this stage was lower. 3) The time interval from courtship initiation to insemination was longer in heterotypic encounters than homotypic encounters. In some crosses, males did not attempt to initiate heterotypic encounters, indicating that this sex is responsible for sexual isolation in these instances. We use our results to formulate specific hypotheses concerning the behavioral basis of sexual isolation in these salamanders, and some possible experimental approaches are suggested.

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Given the long history of studies quantifying sexual (or ethological) isolation between strains, races, or species (reviewed in Spieth and Ringo [1983]), it is surprising how rarely observations of courtship interactions between populations have been made (Spieth, 1949, 1968; Koref-Santibanez, 1963, 1972; Sawada, 1963). The immediate benefit of such behavioral observations is to aid in the identification of both the stage(s) of breakdown in courtship encounters and the sex responsible; knowledge of these in turn can lead to tests of hypotheses about the morphological structures and behavior patterns involved in the maintenance of sexual isolation. Thus, the observation of courtship encounters between isolated populations is a necessary precursor to experimental studies of the behavioral basis of sexual isolation. Because quantitative studies of sexual isolation have usually gone no further than the stage of measuring frequencies of within- and between-population matings (without actually observing the animals), we know very little about the role played by sexual behavior in the critical, early stages of speciation events.

We observed sexual behavior in order to resolve the microevolution of sexual isolation in the mountain dusky salamander, *Desmognathus ochrophaeus*. This plethodontid salamander is a montane species ranging in distribution from New York to northern Georgia in eastern North America (Tilley, 1973). Allozyme studies of populations in the southern Appalachian Mountains have revealed considerable intraspecific genetic differentiation, with some pairs of populations exhibiting Nei distances as large as 0.46 (Tilley et al., 1978). As in most salamander species, this differentiation in electrochemical properties of enzymes is not paralleled by obvious and consistent morphological or structural differences among populations (Wake, 1981). Studies of sexual isolation, however, reveal a full spectrum of behavioral compatibilities between individuals from allopatric populations of this species; some pairs of populations are fully sexually compatible, while others are completely incompatible (Houck et al., 1988; S. G. Tilley et al., unpubl.). In contrast to recent findings for *Drosophila* (see Coyne and Orr, 1989), sexual isolation in mountain dusky salamanders is only very weakly related to allozyme differentiation (S. G. Tilley et al., unpubl.).

In this report, we focus on the progress of courtship encounters in pairs composed of animals from different allopatric populations of *Desmognathus ochrophaeus*. We sought to determine the stage(s) at which

TABLE 1. Locality information for the nine populations of *Desmognathus ochrophaeus* from which the animals used in this study were collected.

Code	Locality (county, state)	Designation in Tilley et al. (1978)
MR	Mount Rogers (Grayson, VA)	2
UN	Crest of Unaka Mountains (Unicoi, TN)	6
MM	Mount Mitchell (Neal's Creek) (Yancey, NC)	9
WR	Waterrock Knob in Plott Balsam Mountains (Haywood-Jackson, NC)	15
RB	Rough Butt Bald in Great Balsam Mountains (Jackson, NC)	17
HP	Whiteside Mountain on Highlands Plateau (Jackson, NC)	21
WA	Wayah Bald in Nantahala Mountains (Macon, NC)	26
SI	Deep Gap under Standing Indian Mountain (Macon, NC)	28
JK	John's Knob in Unicoi Mountains (Graham, NC)	30

courtships break down so that we could design experiments that will help identify the behavioral causes of sexual isolation. We made observations of sexual behavior in multiple crosses between populations so that we could determine whether courtship breaks down at different stages in different crosses and whether the characteristic stage of breakdown was related to the degree of sexual isolation.

MATERIALS AND METHODS

Subjects.—Individuals of *Desmognathus ochrophaeus* were collected in August 1986 and May 1987 from allopatric populations at nine localities in the southern Appalachian mountains. Seven localities were in North Carolina, one was in Tennessee, and one was in Virginia; these are the same sites designated as localities 2, 6, 9, 15, 17, 21, 26, 28, and 30 by Tilley et al. (1978) (see Table 1 for details). Between 12 and 24 adult individuals of each sex were collected from each locality. All of the salamanders used in our experiments were judged to be sexually mature and in reproductive condition: in females, large yolk-filled oocytes were clearly visible through the body wall; in males, the premaxillary teeth, the mental gland on the chin, and clusters of cloacal glands were well-developed (Sever, 1976).

Maintenance.—Animals were kept at a temperature of 15° ± 1°C on a natural (Chicago) photoperiod. Salamanders were housed individually in plastic boxes measuring 9 × 17 × 31 cm, with a damp paper towel as a substrate and a crumpled damp towel as a refuge. Each box contained at least two culture vials of *Drosophila,* which were replaced with sufficient frequency to ensure ad libitum access to larval and adult flies for food.

General Experimental Design.—Ten males and 10 females were used from each population in each cross between a pair of allopatric salamander populations. If the symbols *A* and *B* represent the two populations in a cross (Fig. 1), these 40 salamanders comprised: 1) 10 females of *A,* numbered 1–5 (labelled *A*) and 6–10 (*A**); 2) 10 males of *A,* numbered 1–5 (*A*) and 6–10 (*A**); 3) 10 females of *B,* numbered 1–5 (*B*) and 6–10 (*B**); and 4) 10 males of *B,* numbered 1–5 (*B*) and 6–10 (*B**).

Heterosexual encounters between these salamanders were staged (Fig. 1). For example, *A* females 1–5 encountered *A* males 1–5 on the first and third nights of the cross and *B* males 1–5 on the second and fourth (final) nights. Similarly, *A** females 6–10 encountered *B** males 6–10 on nights 1 and 3 and *A** males 6–10 on nights 2 and 4. Individual females were randomly assigned partners from the appropriate block of males, except that no individual males and females encountered one another more than once. Therefore, all encounters within a cross were unique, although each individual salamander was used multiple times. Individuals were kept in solitary confinement for at least three nights between successive encounters. Both males and females are able to sustain high levels of sexual activity for long periods (several months) if at least three nights of solitude are allowed between successive courtship encounters (Houck et al., 1985; Verrell, 1988*a*, 1988*b*). The first crosses were initiated in November 1986, and the last were initiated in June 1987.

Although spermatophore deposition and insemination rates show some seasonal variation in within-population sexual encounters (Houck et al., 1985), measures of sexual isolation apparently are seasonally robust (S. G. Tilley et al., unpubl.). Mating frequencies in this species remain constant over a range of different conditions in the laboratory, i.e., in sexual encounters staged in courtship boxes differing in size and contents (Verrell, 1988*c*).

Within each cross, there were four different types of pairings: male *A* × female *A*, male *B* × female *B*, male *A* × female *B* and male *B* × female *A*. Twenty unique male-female encounters were staged within each type of pairing, yielding a total of 40 overall within-population (homotypic) encounters ([*A* × *A*] + [*B* × *B*]) and 40 overall between-population (heterotypic) encounters ([*A* × *B*] + [*B* × *A*]).

Crosses.—The following crosses between allopatric salamander populations were conducted (see Table 1 for key to abbreviations): 1) RB × HP (November–December 1986); 2) MM × HP (December 1986–January 1987; 3) UN × JK (December 1986–January 1987); 4) RB × SI (January 1987); 5) UN × MR (January–February 1987); 6) RB × UN (March–April 1987); 7) HP × MR (March–April 1987); 8) UN × SI (April 1987); 9) SI × WR (June 1987); 10) SI × WA (June 1987); and 11) MM × UN (June 1987).

To stage an encounter, a single male and female were placed together in a clean, transparent plastic box (measuring 9 × 17 × 31 cm), with a damp paper towel as a substrate: no refuge was provided. The individuals were introduced at approximately 6:00 P.M., at which time the main white lights in the laboratory were extinguished and replaced by dim red lights (these salamanders are nocturnally active).

Courtship behavior and Courtship Scores.—Courtship in *D. ochrophaeus* follows a temporal scheme that is characteristic of all of the diverse tribes of plethodontid salamanders (Arnold, 1976, 1977; see also Organ [1961] and Arnold and Houck [1982]). A sexually active male repeatedly pursues a moving female and then approaches her very slowly in a crouched posture, from a distance equivalent to about one body length. The male tends to move toward the female's head and then gently nudges her with his snout. Females often move away from the male at this stage, in which case he reverts to active pursuit followed by a slow, crouching approach. If the female remains stationary once the male contacts her head, a bout of head-rubbing may ensue. In such a bout, the male first repeatedly rubs his snout on the lateral portion of the female's head. At irregular intervals during head-rubbing, the male may then lift his head under the female's chin, forcing her head upwards. As before, the female may move away from the male, causing him to revert to pursuit and approach behavior.

An invitation to tail-straddling walk, the precursor to sperm transfer, begins when the

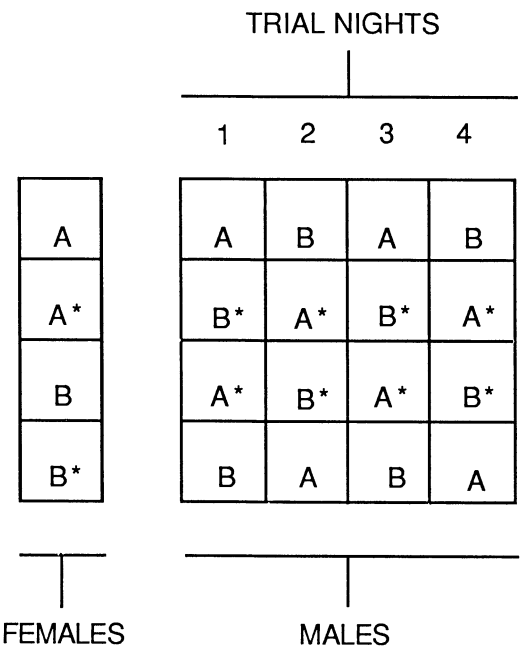

FIG. 1. Diagrammatic representation of the experimental design employed in this study, which comprises an incomplete Latin square. *A* and *B* refer to allopatric populations of salamanders from which 10 individuals of each sex were collected (individuals 1–5 labelled *A* and *B*; individuals 6–10 labelled *A** and *B**). Individuals from each population experienced within-population and between-population heterosexual encounters over four nights according to the schedule depicted (with three nights of solitude between each encounter night). Further details of this design are given in the text.

male ceases head-rubbing and slides forward under the female's chin. The male lifts his body, holding it in contact with the female's chin as he moves forward. He moves the anterior part of his body in a circle, placing his chin on the dorsum of the female just as his dorsal tail base contacts her chin. Holding this configuration, the male laterally undulates his tail base while pressing the tip of his chin downward against the female's back. The male may now engage in two kinds of forceful head movements. He may repeatedly pull his head backwards a few millimeters apparently rasping his protruding premaxillary teeth on the female's back and then slide his head forward again. The male may also vigorously snap his head downward and backward in a rapid action that flings his head (and sometimes his whole body) away from the female. Pulling and snapping are believed to result in the introduction of secretions of the male's mental (chin) gland into the female's peripheral circulation (Organ, 1961; Arnold and Houck, 1982). These secretions increase the rate at which females become fully sexually responsive (Houck, 1986; L. Houck and N. Reagan, unpubl.).

If the female has stepped astride the male's tail, he may move forward in a tail-straddling walk that aligns the bodies of both animals. Otherwise, the male reverts to pulling and snapping or, if the female moves away, to pursuit and approach. After a tail-straddling walk, during which the pair moves forward about one body length or less, the male stops and deposits a spermatophore. During deposition, the female remains astride the male's tail with her chin resting on the dorsum of his gently undulating tail base. The male requires a few minutes to attach the base of his spermatophore to the substrate. After deposition, he moves forward, lifting his vent from the affixed spermatophore and pulling his tail off to one side. The female now has an unobstructed path to the spermatophore, and she moves forward and over it, all the while keeping her chin on the male's raised tail base. She settles over the spermatophore when it contacts her vent and remains with the uppermost sperm-filled cap inserted into her vent for a few minutes. Meanwhile, the male stops in front of the female; he now raises and lowers his tail base under the female's chin by flexing and extending his hind limbs. Courtship ends when the female, perhaps with the sperm cap lodged in her vent, moves away from the male. It is most usual for only one spermatophore to be deposited in an evening (Houck et al., 1985; Verrell, 1988*b*). Spermatophore deposition does not occur in the absence of the tail-straddling walk, but not all episodes of tail-straddling walk result in deposition.

During observation sessions, the behavior of pairs of salamanders was scored on a qualitative scale along the courtship sequence. At the lower end of the scale, 0 represents no physical contact between the partners; the salamanders appear to ignore one another. The next score, 1, corresponds to active pursuit of the female by the male or simple physical contact between the partners in the absence of any specific behavioral interactions (e.g., when the salamanders lie next to one another). We refer to this as the "pursuit stage" of courtship. A score of 2 corresponds to the initiation of courtship behaviors involving intimate physical contact between the stationary partners (head rubbing, head lifting, pulling, and snapping). Because secretions delivered by the male during pulling and snapping are believed to increase the sexual responsiveness of the female, we refer to this as the "persuasion stage" of courtship. The highest score in the scale, 3 represents the observation of attempted sperm transfer, during which time the partners move in a straight line during the tail-straddling walk (the "sperm transfer stage" of courtship).

The behavior of each pair of salamanders was recorded according to the scan-sampling protocol described by Altmann (1974). After a settling period of 90 minutes (i.e., beginning at 7:30 P.M.), all of the courtship boxes were visually scanned, and the behaviors of the pairs were scored as described above. This scanning procedure was repeated at the end of consecutive 30-minute time periods until approximately midnight. In this way, a record of the behavior of each pair was obtained for ten instantaneous moments during the observation session. Analysis of videotaped sexual encounters indicated that, due to the slow pace at which courtship proceeds, a scan resolution of 30

minutes was sufficient for the courtship stages to be observed.

At the end of the observation session, the scores recorded for all pairs were examined, and two further scores were derived: 1) the maximum score obtained by a pair during the session and 2) the time taken for the pair to attain the persuasion stage of courtship (stage 2), assuming that the pair progressed this far. If this stage of courtship was not observed but sperm transfer behavior was seen, a stage 2 was assumed to have occurred during the previous 30-minute period.

Pairs were left together overnight and examined the following morning, at approximately 8:00 A.M. The cloaca of the female was examined for the presence of a white sperm mass, a certain indicator of successful insemination. This mass remains externally visible for as long as 24 hours after mating (Houck et al., 1985). In addition, the presence and number of spermatophore bases on the towel substrate were recorded. Between subsequent courtship encounters, the salamanders were returned to their own maintenance boxes for their nights of solitude.

RESULTS

Sexual Responsiveness and Sexual Isolation.—Table 2 presents data on the numbers of spermatophores deposited by males and picked up by females. Mating propensities were high during the course of the study (i.e., salamanders of both sexes were sexually responsive; column 1 of Table 2). For spermatophore deposition (regardless of insemination success), an average of 71% of homotypic encounters resulted in at least one deposition, compared with 31% of heterotypic encounters. On average, 64% of homotypic encounters resulted in insemination, compared with 25% of heterotypic encounters. Paired t tests were performed on the data in Table 2 to test the null hypothesis that mating was equally likely to occur in homotypic and heterotypic encounters. Significant differences were evident for both spermatophore deposition ($t = 9.18$, $P < 0.01$) and sperm-mass pick up ($t = 12.38$, $P < 0.001$).

Data on the numbers of inseminations obtained during homotypic and heterotypic encounters can be used to calculate an overall index of the strength of sexual isolation between individuals sampled from two populations. Indexes of sexual isolation were obtained using the following formula derived by Stalker (1942):

$$\frac{\text{number of homotypic matings} - \text{number of heterotypic matings}}{\text{number of homotypic matings} + \text{number of heterotypic matings}}.$$

This formula yields an index of sexual isolation which ranges from −1 (only heterotypic matings), through 0 (random mating) to 1 (only homotypic matings and, thus, complete sexual isolation). The isolation indexes calculated from insemination data for the 11 crosses (Table 2) ranged from 0.26 to 0.82 (the weakest and strongest isolation indices, respectively). The ranking of sexual isolation indexes for all crosses calculated from insemination data is positively correlated with the ranking calculated from spermatophore-deposition data (Spearman's $r = 0.86$, $P < 0.01$). In all subsequent tables, the order in which crosses are listed corresponds to their relative ranking in terms of the strength of sexual isolation calculated from insemination data (always ranked from weakest isolation to strongest).

TABLE 2. Courtship success in pooled homotypic pairings and pooled heterotypic pairings involving nine allopatric populations of *Desmognathus ochrophaeus*. The number of spermatophore depositions in 40 trials is shown in the first two columns, followed by the number of inseminations (in parentheses). Stalker's isolation index for spermatophore-deposition scores is shown in the last column (index for insemination scores in parentheses).

Cross	Courtship success: Homotypic pairings ($N = 40$)	Courtship success: Heterotypic pairings ($N = 40$)	Isolation index
MM × UN	30 (29)	19 (17)	0.22 (0.26)
MM × HP	29 (26)	22 (12)	0.14 (0.37)
SI × WR	32 (28)	12 (12)	0.45 (0.40)
RB × UN	37 (30)	15 (12)	0.42 (0.43)
RB × SI	29 (28)	11 (11)	0.45 (0.44)
SI × WA	24 (21)	10 (8)	0.41 (0.45)
RB × HP	22 (19)	9 (5)	0.42 (0.58)
UN × SI	24 (21)	7 (5)	0.55 (0.62)
UN × MR	28 (27)	6 (6)	0.65 (0.64)
UN × JK	27 (23)	4 (4)	0.74 (0.70)
HP × MR	33 (31)	3 (3)	0.83 (0.82)

TABLE 3. Courtship-progression scores in 11 crosses composed of sexual encounters within and between nine allopatric populations of *Desmognathus ochrophaeus*. The numbers of encounters (out of a total of 20) that progressed to each of four courtship stages are indicated.

Cross		Number of encounters progressing to stage				
Male	Female	Stage 0	Stage 1	Stage 2	Stage 3	t
MM	MM	2	4	4	10	2.08*
MM	UN	9	3	2	6	
UN	UN	3	6	7	4	4.43**
UN	MM	16	2	0	2	
MM	MM	2	3	4	11	1.86*
MM	HP	6	4	6	4	
HP	HP	4	3	4	9	0.63
HP	MM	4	5	4	7	
SI	SI	2	1	9	8	2.93**
SI	WR	9	6	2	3	
WR	WR	1	3	7	9	6.15***
WR	SI	12	4	3	1	
RB	RB	1	5	5	9	7.57***
RB	UN	6	8	6	0	
UN	UN	2	2	3	13	3.21**
UN	RB	9	3	3	4	
RB	RB	5	4	6	5	2.13*
RB	SI	9	6	5	0	
SI	SI	5	4	5	6	0.15
SI	RB	7	4	2	7	
SI	SI	4	3	7	6	3.33***
SI	WA	14	4	1	1	
WA	WA	3	6	6	5	1.87*
WA	SI	7	5	5	3	
RB	RB	5	5	4	6	4.27**
RB	HP	16	3	1	0	
HP	HP	7	2	5	6	3.48**
HP	RB	12	4	4	0	
UN	UN	6	4	4	6	3.75**
UN	SI	9	8	3	0	
SI	SI	3	6	5	6	3.07**
SI	UN	9	6	3	2	
UN	UN	4	5	5	6	3.47**
UN	MR	9	9	1	1	
MR	MR	2	4	5	9	5.76***
MR	UN	8	9	3	0	
UN	UN	2	6	7	5	3.85**
UN	JK	9	5	6	0	
JK	JK	7	4	6	3	2.24*
JK	UN	8	9	3	0	
HP	HP	2	6	2	10	2.33*
HP	MR	4	7	8	1	
MR	MR	4	2	5	9	5.38***
MR	HP	12	7	1	0	

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Courtship Progression.—Scan-sampled behavioral data for all pairings of 11 crosses are presented in Table 3. Within each type of pairing, the scores for progression along the courtship sequence for each pair of sexual partners were extracted and summed. Paired t tests were used to test the null hypothesis that, for each population pair, progression along the courtship sequence did not differ between homotypic and heterotypic encounters involving males from the same population. Significant differences in progression along the courtship sequence were found for all pairs of populations observed (Table 3). In all but two crosses, significant differences were evident in both reciprocal comparisons.

These data indicate that heterotypic encounters do not progress as far along the courtship sequence as do homotypic encounters. Table 3 shows that heterotypic encounters ended at varying stages along the courtship sequence. For the nine crosses in which both reciprocal heterotypic pairings were significantly different from their respective homotypic pairings ($N = 18$ homotypic and $N = 18$ heterotypic pairings), an average of 17% of the homotypic encounters failed to result in any sign of sexual activity (stage 0), 20% progressed only to the pursuit stage (stage 1), 27% progressed to the persuasion stage (stage 2), and 36% progressed as far as the spermatophore-transfer stage (stage 3). For the heterotypic pairings, the respective average values are 48% (stage 0), 28% (stage 1), 17% (stage 2) and 7% (stage 3). These mean values clearly obscure intercross variability in the data, evident from close inspection of Table 3.

In all crosses, males were more likely to progress to the persuasion stage of courtship in homotypic encounters than in heterotypic encounters (paired t test, $t = 48.5$, $P < 0.001$; Table 3). On average, only 44% as many heterotypic encounters progressed this far. Variability was not significantly greater in heterotypic encounters (variance = 20.7) than in homotypic encounters (variance = 15.6) ($F_{[1,42]} = 1.33$, $P > 0.05$). To examine the relationship between the strength of sexual isolation and the stage of courtship breakdown, isolation indexes were correlated with the numbers of heterotypic encounters progressing to stages 2 and 3 of

courtship for all 11 crosses. Although the resulting correlation is negative (Spearman's $r = -0.49$), indicating that high indexes were associated with low frequencies of courtship progression, it is not statistically significant ($P > 0.05$).

Time to Courtship Onset.—For the purposes of this analysis, a pair of salamanders was regarded as engaged in courtship if they were observed in either the persuasion or sperm-transfer stages during an observation session (stages 2 and 3 in Table 3). For each pair of populations, time taken to achieve engagement in courtship was determined separately for each of the two homotypic pairings and for each of the two heterotypic pairings (due to the nature of the scan-sampling protocol, there is a 30-minute resolution on these times). Then, for each of these four types of pairings, mean times to achieve engagement in courtship were calculated, provided that the number of time scores for a pairing numbered five or more.

For 11 (50%) of all heterotypic pairings (Table 4), it was impossible to compute accurate estimates of mean time scores because there were too few encounters in which stages 2 or 3 of courtship were achieved during observation sessions (i.e., $N < 5$ encounters). Two statistical tests were employed to test the null hypothesis that homotypic and heterotypic pairings did not differ in time taken to initiate courtship. In the first, the median times to courtship initiation for the 22 homotypic and 11 heterotypic pairings were compared. These values are 256.0 minutes (range = 180.0–365.0 minutes) and 267.2 minutes (range = 178.1–310.0 minutes), respectively. This difference is not significant (Mann-Whitney U test for large samples, $z = 0.52$, $P > 0.5$). In the second test, time scores within the same type of pairing in each cross were averaged and then compared across pairing types (homotypic vs. heterotypic). Once again, no significant difference was found (paired t test, $t = 0.07$, $P > 0.05$).

The Timing of Spermatophore Deposition.—Although observation sessions terminated at midnight, pairs were not separated at this time and were therefore able to court during the remainder of the night. Behavioral observation until midnight and examination of the courtship boxes and females the next morning made it possible to determine whether courtships resulting in spermatophore deposition (with or without insemination) occurred early (before midnight) or late (after midnight) during the total period available for courtship and mating.

The number of encounters resulting in spermatophore deposition during observation sessions may be expressed as a percentage of the total number of depositions that were recorded on morning examination (this total includes those depositions occurring both during and after observation sessions). In all but one cross, a higher percentage of spermatophore depositions was observed in homotypic pairings than in heterotypic pairings (in the one exceptional cross (RB × SI) the percentages of sperm depositions were 37.9% [homotypic] and 63.6% [heterotypic]). This pattern is statistically significant over the 11 pairs of populations crossed (paired t test, $t = 3.25$, $P < 0.025$). On average, 50% of all homotypic spermatophore depositions were observed before midnight, compared with only 32% of all heterotypic depositions (ranges = 29.6–68.9% and 0–63.6%, respectively).

Discussion

Our results clearly demonstrate that differences in sexual behavior between populations may evolve during the time that they experience differentiation in allopatry. The indexes of sexual isolation obtained for crosses between populations of *Desmognathus ochrophaeus* ranged from 0.26 (MM × UN) to 0.82 (HP × MR), i.e., from almost complete random mating to almost complete homotypic mating. Evolutionary forces that may drive behavioral divergence in allopatric populations include genetic drift and founder events, local adaptation, and sexual selection (Muller, 1942; Mayr, 1963; Lande, 1981, 1982; Paterson, 1985; Verrell, 1988*d*). Our results support the hypothesis that mechanisms involved in sexual isolation between populations begin their divergent evolution before secondary contact is established (Verrell, 1988*d*). Sexual isolation evolves gradually in allopatric populations and apparently involves numerous behavioral mechanisms. The relationships between sexual isolation (based on insem-

TABLE 4. Time to onset of courtship in 11 crosses within and between nine allopatric populations of *Desmognathus ochrophaeus*.

Homotypic crosses				Heterotypic crosses			
Male	Female	*N*	Mean time to onset of courtship (min)	Male	Female	*N*	Mean time to onset of courtship (min)
MM	MM	14	229.3	MM	UN	8	178.1
UN	UN	11	271.4	UN	MM	2	—
MM	MM	15	288.0	MM	HP	10	279.0
HP	HP	13	285.0	HP	MM	12	295.0
SI	SI	17	255.9	SI	WR	6	310.0
WR	WR	16	254.0	WR	SI	4	—
RB	RB	14	270.0	RB	UN	6	250.0
UN	UN	16	241.9	UN	RB	8	270.0
RB	RB	11	234.0	RB	SI	4	—
SI	SI	11	256.4	SI	RB	9	300.0
SI	SI	13	240.0	SI	WA	2	—
WA	WA	11	268.6	WA	SI	8	264.4
RB	RB	10	291.0	RB	HP	1	—
HP	HP	11	316.4	HP	RB	4	—
UN	UN	10	258.0	UN	SI	3	—
SI	SI	11	180.0	SI	UN	5	252.0
UN	UN	12	252.5	UN	MR	2	—
MR	MR	14	256.1	MR	UN	3	—
UN	UN	13	361.0	UN	JK	6	307.5
JK	JK	9	365.0	JK	UN	3	—
HP	HP	11	256.4	HP	MR	9	260.0
MR	MR	14	342.8	MR	HP	1	—

ination frequencies) and geographic and genetic distances between allopatric *D. ochrophaeus* populations will be considered elsewhere, using a larger data set (S. G. Tilley et al., unpubl.).

The behavioral data we obtained by scan-sampling do not provide fine resolution but are sufficiently detailed to reveal that, on average, heterotypic encounters fail to progress as far along the courtship sequence as homotypic encounters; 64% of all homotypic encounters progressed to the persuasion stage of courtship, compared with only 26% of heterotypic encounters. Also, these data suggest that heterotypic encounters may become blocked at different stages of courtship (Table 3). In some crosses, a marked asymmetry exists in the extent to which heterotypic pairings progressed along the courtship sequence (Table 3). For example, in the MM × UN cross, MM males progressed to the persuasion stage of courtship with UN females in 40% of encounters, whereas UN males progressed to this stage with MM females in only 10% of encounters. Similarly, a score of 0 (no interest) was recorded in 80% of UN male × MM female encounters, but in only 45% of MM male × UN female encounters. How such asymmetries are related to behavioral differences between populations awaits further investigation.

More homotypic than heterotypic encounters resulted in courtship progressing to the persuasion stage. However, heterotypic encounters that attained this stage did so with the same speed as homotypic encounters (mean times of 267 minutes and 256 minutes, respectively). It appears that courtship never gets underway in a fraction of the encounters, apparently because males ignore females; however, for those males that do initiate courtship, the temporal aspects of early courtship behavior appear to be roughly the same as those for homotypic encounters (Table 4).

The ultimate measure of courtship success is the probability that the female will

become inseminated. However, the level of illumination during observation periods was insufficient to determine whether a female had picked up a sperm mass, and it was possible to determine only spermatophore deposition. Considering these data for spermatophore deposition (Table 2), it is clear that fewer depositions occurred during heterotypic encounters than during homotypic encounters over the course of the entire night. Furthermore, the average percentage of all heterotypic depositions that occurred before midnight (32%) was lower than the percentage of homotypic depositions (50%). This result suggests that, even though heterotypic encounters may reach the persuasion stage at the same time as homotypic courtships, the interval from persuasion to deposition is longer in the heterotypic encounters (given that deposition will occur at all). The nonsignificant negative correlation obtained between sexual isolation indices and numbers of heterotypic encounters progressing to courtship stages 2 and 3 suggests that the stage of courtship breakdown is a poor predictor of the strength of sexual isolation in the 11 crosses conducted.

The only detailed study of behavioral aspects of sexual isolation published for other urodele amphibians is for Japanese newts of the genus *Cynops*. Kawamura and Sawada (1959) and Sawada (1963) observed sexual encounters between individuals of various allopatric races of *C. pyrrhogaster* and *C. ensicauda*. In general, males initiated courtship with heterotypic females, but females failed to become fully sexually responsive. This may have been due to geographic differences in male display posture during the early stages of courtship (see Kawamura and Sawada, 1959 fig. 4). In those heterotypic encounters in which courtship progressed as far as the spermatophore-transfer stage, insemination was seldom successful, either due to the presence (or absence) of specific behavioral elements or due to differences in the rates of movement of the two partners. Two major conclusions are evident from this work. First, mechanisms that confer sexual isolation between different populations of newts apparently have evolved during divergence in allopatry. Second, male newts contribute less to the maintenance of sexual isolation than do females, at least during the earlier stages of courtship.

The results obtained for *Cynops* can be compared with those from our study. The most obvious similarity is that mechanisms conferring sexual isolation apparently have evolved in allopatry in both groups. However, the data differ in that, in general, male *Desmognathus* appear to contribute more to the maintenance of sexual isolation than do male newts. Male *Desmognathus* commonly do not even initiate courtship encounters with heterotypic females (as is also the case for interspecific encounters in *Desmognathus* [Verrell, 1989] and *Plethodon* [Dawley, 1986]). We are currently undertaking a detailed video study of geographic differences in courtship behavior in *D. ochrophaeus* and will document our results in a later report. Our initial impression is that geographic differences mostly involve differences in the timing of courtship.

Sexual isolation has been most extensively investigated in the genus *Drosophila*. However, as noted almost 40 years ago by Spieth (1949; also see Spieth and Ringo [1983]), direct observations of heterotypic pairings have been reported only rarely. Most studies of *Drosophila* have used either "no choice" or "multiple choice" protocols in which data on frequencies of insemination are used to calculate sexual-isolation indexes. Other studies have surveyed and described patterns of homotypic courtship behavior and then inferred that behavioral differences between strains, races, or species might be important as sexual isolating mechanisms (e.g., Ringo and Hodosh, 1978; Oguma et al., 1987).

Detailed behavioral observations of heterotypic pairings are available for *Drosophila* in the *virilis* group (Stalker, 1942; Spieth, 1951), the *willistoni* group (Spieth, 1949), the *mesophragmatica* group (Koref-Santibanez, 1963), the *paulistorum* group (Koref-Santibanez, 1972), and the *melanogaster* group (Wood and Ringo, 1980; Cobb et al., 1988). Few generalizations can be made about the results of these studies. In the *willistoni* group, males will often initiate courtship with heterotypic females but then terminate their efforts before intromission (in at least 12 out of 30 crosses); in a smaller number of crosses, females definitely were

responsible for the termination of courtship. In the *mesophragmatica* and *paulistorum* groups, many males do not even initiate heterotypic courtships; when heterotypic courtships occur, they tend to be of shorter duration than homotypic courtships. In the *virilis* and *melanogaster* groups, sexual isolation may be due to discrimination against heterotypic females by males. However, in some crosses, discrimination may be exercised by females and may apparently act in concert with interspecific differences in mating propensity to produce sexual isolation.

Observations of courtship-behavior patterns in heterotypic crosses between *Desmognathus ochrophaeus* populations can be used to test the plausibility of hypotheses concerning the behavioral basis of sexual isolation. For example, the failure of males to pursue heterotypic females suggests that there may be disruption of the chemosensory systems involved in the recognition of appropriate mating partners. Dawley (1984, 1986) provides evidence that odors may be important in the maintenance of sexual isolation between species in the salamander genus *Plethodon*. This hypothesis could be tested in *D. ochrophaeus* using the Y-maze olfactometer device designed by Dawley (interspecific differences in female olfactory cues may be responsible for the maintenance of sexual isolation between *D. ochrophaeus* and the related salamander *D. imitator* at a locality where they are sympatric [Verrell, 1989]). When the male pursues a heterotypic female but fails to engage her in the persuasion stage of courtship, the female may repeatedly flee from the male because he is too small or too large (mean body size shows considerable variation between populations [Verrell, unpubl.]), because his odor is inappropriate, or because he gives an inappropriate display during pursuit. Courtship breakdown after the persuasion stage may occur because the male delivers an inappropriate mental-gland secretion during snapping and pulling, because he has premaxillary teeth of an inappropriate morphology, because he has an inappropriate temporal pattern of secretion delivery, or because his behavior during the sperm-transfer stage of courtship is inappropriate. Simple observations of courtship progression have enabled us to pose specific questions about why heterotypic courtships terminate prematurely or proceed slowly. Our next challenge is to answer these questions using detailed observational and experimental methods.

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