# Sexual compatibility and male olfactory discrimination in two populations of *Eurycea bislineata* (Green), the two lined salamander, in Ohio.

by

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## ABSTRACT

Sexual compatibility and male olfactory discrimination in two populations of *Eurycea bislineata* (Green), the two lined salamander, in Ohio.

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Master of Science

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The taxonomic status of *Eurycea bislineata,* the two-lined salamander, in Ohio is equivocal. Recently, Guttman (1989) suggested that an investigation of reproductive isolating mechanisms might aid in clarifying the status of two possible cryptic species in eastern Ohio.

Two sets of experiments were performed using members of two populations of salamanders considered to represent Guttman's cryptic species. Intrapopulational and interpopulational sexual encounters were arranged resulting in the conclusion that the populations are conspecific. Results of male olfactory discriminatory tests reinforced the single species concept.

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# INTRODUCTION

Ernst Mayr (1942) defined a biological species as a group of organisms that interbreed or are capable of interbreeding and producing fertile offspring, however, are reproductively isolated from other related organisms. For centuries before this, biologists struggled with the confusing picture that often emerged when only visible phenotypic variability was examined. We now know that such variability is widespread in organisms, but not necessarily useful in making clear what constitutes a species. Since Mayr's monumental theory was proposed, considerable research has been devoted to discovering the nature of factors that act as reproductive isolating mechanisms.

A reproductive isolating mechanism is a factor that blocks or inhibits gene exchange between two populations of an organism. Further, we have come to realize that an isolating mechanism may play a role prior to sexual union and is then known as a pre-mating mechanism or it may function after mating has occurred and then is called a post-mating isolating mechanism. Pre- mating mechanisms are those that prevent interspecific crosses from occurring and may include ecological factors such as habitat preference or time of breeding (seasonal), breeding behavior, inability to recognize a member of the opposite sex as a potential mate or a sexual mechanical incompatibility. Post-mating mechanisms include gametic incompatibility, hybrid sterility, hybrid breakdown, and other factors. Both premating and postmating factors reduce or prevent gene flow between two species and protect the integrity of a gene pool.

Recently, considerable interest among herpetologists has centered on unraveling courtship behavior sequences of salamanders (Uzendoski and Verrell 1993, Houck et al. 1988, Verrell 1988a). Successful courtship is essential to effect internal fertilization among salamanders. Among species of the Family Plethodontidae, the lungless salamanders, courtship is complex and often lengthy. A female and male must cooperate throughout the courtship and if there are breakdowns along the way the courtship will end. A typical courtship may have many physical cues recognized by each sex as the standard protocol for an intraspecific breeding. Recognition of differences during courtship by mating partners may play an important role as pre-mating isolating mechanisms (Uzendoski and Verrell 1993).

There are also species specific chemical cues that are involved before and during the courtship (Dawley 1986). A salamander for example, must be able to identify a conspecific mate in order to successfully breed. By correctly recognizing conspecific cues, time and energy is not wasted on a heterospecific mate. Although statistically unlikely that two different species could transfer a sperm packet due to the elaborate courtship behaviors and chemical cues involved, however, it does occur between *Desmognathus ochrophaeus* and *D. fuscus* (Houck et al.1988)

The lungless salamanders are among the most successful families of salamanders. Within this family are a number of genera in which clusters of closely related species have evolved. Members of the genus *Desmognathus* are notorious for their morphological similarities, e.g., *D. fuscus and D. ochrophaeus* (Karlin and Pfingsten, 1989; Orr, 1989).

The two-lined salamander, *Eurycea bislineata* (Green), a member of the Plethodontidae, is widely distributed in eastern North America. Various interpretations of this wide ranging species exist. One is that several subspecies exist (Mittleman, 1949, 1966; Guttman, 1989). Mittleman subdivided Ohio *E. bislineata* into two subspecies, *E. b. bislineata*, the northern two-lined salamander and *E. b. rivicola*, the midwest two-lined salamander, occurring in the Ohio River Valley. Sever (1972), after conducting a detailed study of morphological variability in *E. bislineata*, concluded that Ohio salamanders are members of the subspecies, *E. b. bislineata*. After examination of samples from populations from Ohio using starch gel electrophoretic techniques to detect enzyme differences, Guttman and Karlin (1986) suggested that two-lined salamanders in Ohio represent two cryptic species.

The northern species is alleged to occur north of 40° 20' and the southern species south of 40° 00' north latitude (Guttman, 1989). Jacobs (1987) included the southern populations within *E. cirrigera*, a new species created by elevation of the subspecies, *E. b. cirrigera,* based on electrophoretic data. Guttman (1989) suggested that research efforts be directed toward detection of pre- and post-mating isolating mechanisms operating between the two proposed cryptic species.

The present study was designed to examine two pre-mating isolating mechanisms that might shed some light on the taxonomy of *E. bislineata* in Ohio. Since courtship behavior appears to play a pivotal role in the process of insemination among plethodontid salamanders, intrapopulational and interpopulational encounters were observed. The second set of experiments were designed to determine the ability of male salamanders in both populations to detect conspecific females.

## MATERIALS AND METHODS

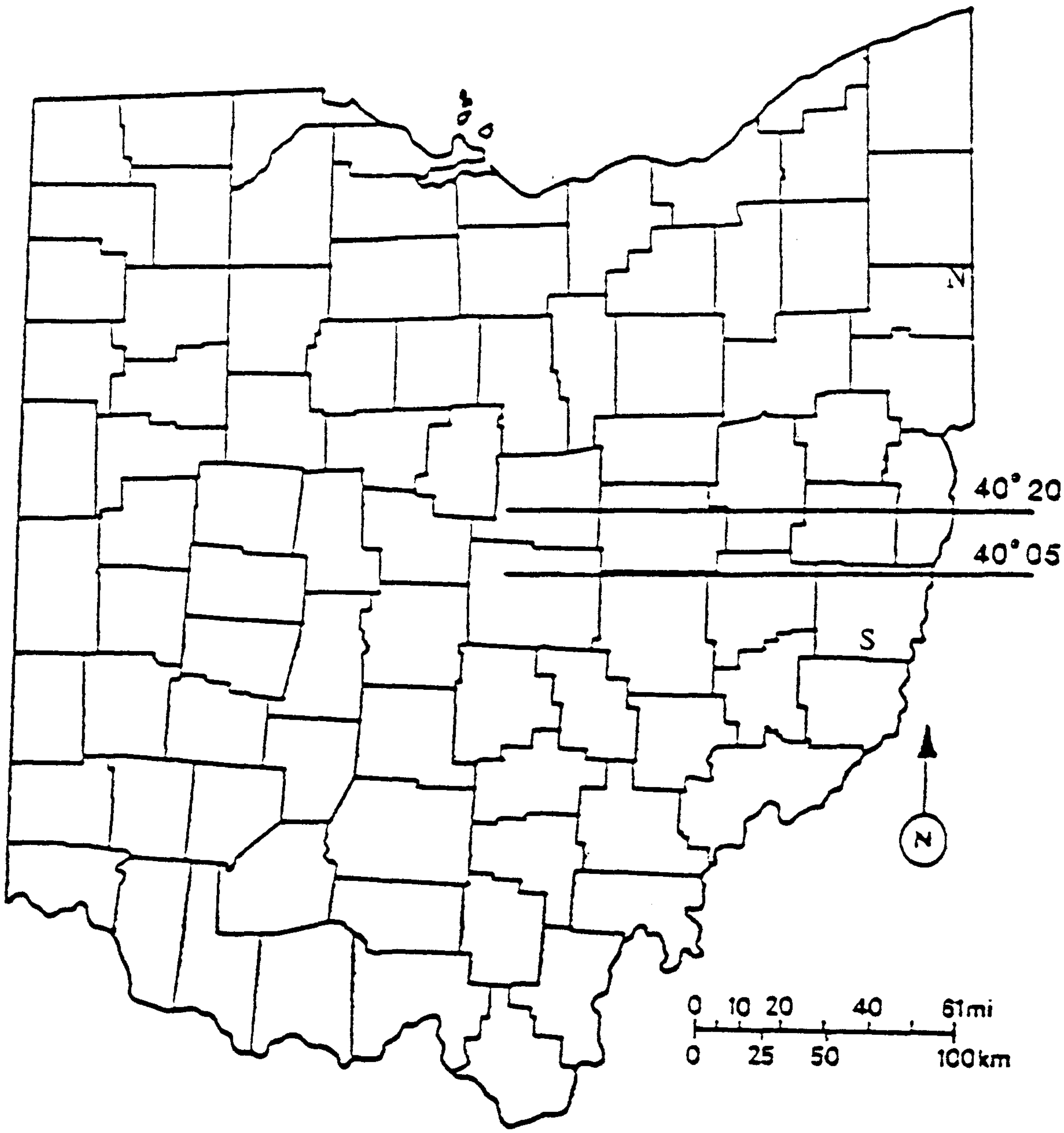
A. Animal Care

In August and September of 1994 salamanders were collected from Gray's Run, Poland TWP, Mahoning Co.($41^{o}$ 02' north latitude), the northern site and Jakes Run, Wayne TWP, Belmont Co.($39^{o}$ 55' north latitude), Ohio the southern site, Ohio by turning over rocks and leaves in the streams. Salamanders from Mahoning Co. were designated as the northern population and those from Belmont Co. were designated the southern population (Fig.1). Only sexually mature specimens were taken. Males, when in reproductive condition possess a tail bump, a mental gland, and cirri, while females possess oocytes that can be seen through the body wall. Only enough salamanders were collected for both the sexual compatibility and the male olfactory discrimination experiments.

Salamanders were transported in cooled containers to the laboratory where they were maintained in programmable walk-in Environmental Growth Chambers. The chamber temperature was set at $15^{o}$ C with an 85% relative humidity. An external (Youngstown Ohio) photoperiod was kept for the duration of the experiments. This period varied along with the changing day lengths for the fall season. A minimum of twelve weeks of acclimation were allowed for salamanders to adjust to the conditions in the containers and environment.

Salamanders were housed individually in 9x17x31cm clear plastic boxes. The bottom was lined with a paper towel and a crumpled paper towel was placed in the box as a refuge. The substrate was kept damp with water from the collecting sites to minimize adjustments to various water conditions, such as pH. Boxes were sectioned off with opaque brown paper which prevented salamanders from seeing their neighbors.

Food provided for the salamanders were cultures of the fruitfly, *Drosophila melanogaster*. Prior to experimental periods the cultures were removed and the salamanders were fed manually to ensure satiation before the test date. A bottle, with a funnel lid was first filled with flies and then placed in an opening of the salamander's container. The tip of the funnel was opened and forty flies were counted as they entered


40° 20
40° 05
N
S
N
0 10 20 40 61mi
0 25 50 100km

the container. Forty flies were considered sufficient since there were many flies left when specimens were removed to use in experiments.

B. Experimental Protocol

Experiment 1. Sexual Compatibility

The experimental design (incomplete Latin-square) followed is similar to that outlined in Tilley et al. (1990). This method allows four types of pairings to be made on the same test date. In the present study both northern and southern populations were tested for reproductive compatibility. The four types of pairings were: (1) northern males vs. northern females, (2) northern males vs. southern females, (3) southern males vs. southern females and (4) southern males vs. northern females.

The design allowed female members of both populations to encounter both northern and southern males during one test date. To simplify record keeping, I substituted letters in place of region of sample origin. The letters NF was assigned to northern females 1-5, NF' was assigned to northern females 6-10, the letters SF, in turn, represents southern females 1-5, and SF', southern females 6-10. Now all 20 females may be thought of as four individual groups, NF, NF', SF and SF', with five organisms in each group. Males were similarly assigned i.e., the 10 northern males as NM 1-5, NM' 6-10 and southern males as SM 1-5 and SM' 6-10.

During the first test night one group of northern females was paired with a group of northern males, (NF:NM) and the second group of northern females was paired with a group of southern males (NF':SM'). Southern females were paired as follows: SF:SM and SF':NM' (Table 1). An examination of Table 1 will reveal alternate pairings for each of the subsequent encounter test dates. A complete pairing system was not used, since it would have consumed considerable time. A northern female, for example, would have met all of the southern and northern males. In order to maintain a high sexual interest, a four night recovery was allowed between sexual encounters (Verrell 1988a). The total time required would have been 96 nights or more than three months. To produce a more

**Table 1.** Trial Schedule of Courtship Encounters for Two Ohio Populations of *Eurycea bislineata,* the Two-lined Salamander.

| | Trial night | | | | | |
|---|---|---|---|---|---|---|
| Source of Females | 12/06/94 | 12/11/94 | 12/16/94 | 12/21/94 | 12/27/95 | 1/04/95 |
| | Males | | | | | |
| NF1 | NM2 | SM2 | NM 3 | SM3 | NM4 | SM4 |
| NF2 | NM3 | SM3 | NM4 | SM4 | NM5 | SM5 |
| NF3 | NM4 | SM4 | NM5 | SM5 | NM1 | SM1 |
| NF4 | NM5 | SM5 | NM1 | SM1 | NM2 | SM2 |
| NF5 | NM1 | SM1 | NM2 | SM2 | NM3 | SM3 |
| NF'6 | SM'7 | NM'7 | SM'8 | NM'8 | SM'9 | NM'9 |
| NF'7 | SM'8 | NM'8 | SM'9 | NM'9 | SM'10 | NM'10 |
| NF'8 | SM'9 | NM'9 | SM'10 | NM'10 | SM'6 | NM'6 |
| NF'9 | SM'10 | NM'10 | SM'6 | NM'6 | SM'7 | NM'7 |
| NF'10 | SM'6 | NM'6 | SM'7 | NM'7 | SM'8 | NM'8 |
| SF1 | NM'7 | SM'7 | NM'8 | SM'8 | NM'9 | SM'9 |
| SF2 | NM'8 | SM'8 | NM'9 | SM'9 | NM'10 | SM'10 |
| SF3 | NM'9 | SM'9 | NM'10 | SM'10 | NM'6 | SM'6 |
| SF4 | NM'10 | SM'10 | NM'6 | SM'6 | NM'7 | SM'7 |
| SF5 | NM'6 | SM'6 | NM'7 | SM'7 | NM'8 | SM'8 |
| SF'6 | SM2 | NM2 | SM3 | NM3 | SM4 | NM4 |
| SF'7 | SM3 | NM3 | SM4 | NM4 | SM5 | NM5 |
| SF'8 | SM4 | NM4 | SM5 | NM5 | SM1 | NM1 |
| SF'9 | SM5 | NM5 | SM1 | NM1 | SM2 | NM2 |
| SF'10 | SM1 | NM1 | SM2 | NM2 | SM3 | NM3 |

NF= northern female population

SF= southern female population

NM= northern male population

SM= southern male population

reasonable schedule, an incomplete pairing design was used. With this technique, a northern female randomly encountered a subset of males from each group, i.e., northern female number one (NF1) was paired with a total of three northern males and three southern males (Table 1). Furthermore, northern female number one (NF1) encountered a northern male on the first, third, and fifth trial dates, and a southern male on the second, fourth, and sixth trial dates.

Courtships were staged in clean boxes of the same type as the housing boxes. A clean paper towel dampened with 20 ml of water was placed on the bottom and no refuge was provided. In an intrapopulational pairing, (NF1:NM2), e.g., 20 ml of water were used from the northern site. In an interpopulational pairing, (NF3:SF'4), e.g., 10 ml from each site were used. Encounters were begun at approximately 1800 under the dark photoperiod. The area was illuminated with six 25 watt, red light bulbs to reduce the sensitivity of the salamanders to movement near them. Courtships were recorded, using a video scanning device built by two Youngstown State University mechanical engineering students, Thomas Persson and Doug Verenski. Only a portion of the night was recorded since eight hour tapes were used. A test for courtship success among plethodontid salamanders for 20 hours following a successful insemination is the presence or absence of a spermatophore in the cloaca of the female (Arnold et al. 1993). Female cloacas were checked in the morning following a sexual encounter. If a white sperm mass was present, the courtship was considered successful.

The data were analyzed using a technique similar to a study by Houck et al. (1988). Merrell's (1950) measure of sexual isolation was used to determine the degree of isolation: I= AB+BA/AA+BB, where AB is the number of successful matings between males from population A (northern) and females from population B (southern). BA was the number of successful matings between males from population B (southern) and females from population A (northern). AA (northern) and BB (southern) were the respective number of successful intraspecific matings. For this index, complete isolation between the populations was represented by I=0 and random mating occurred when I=1.

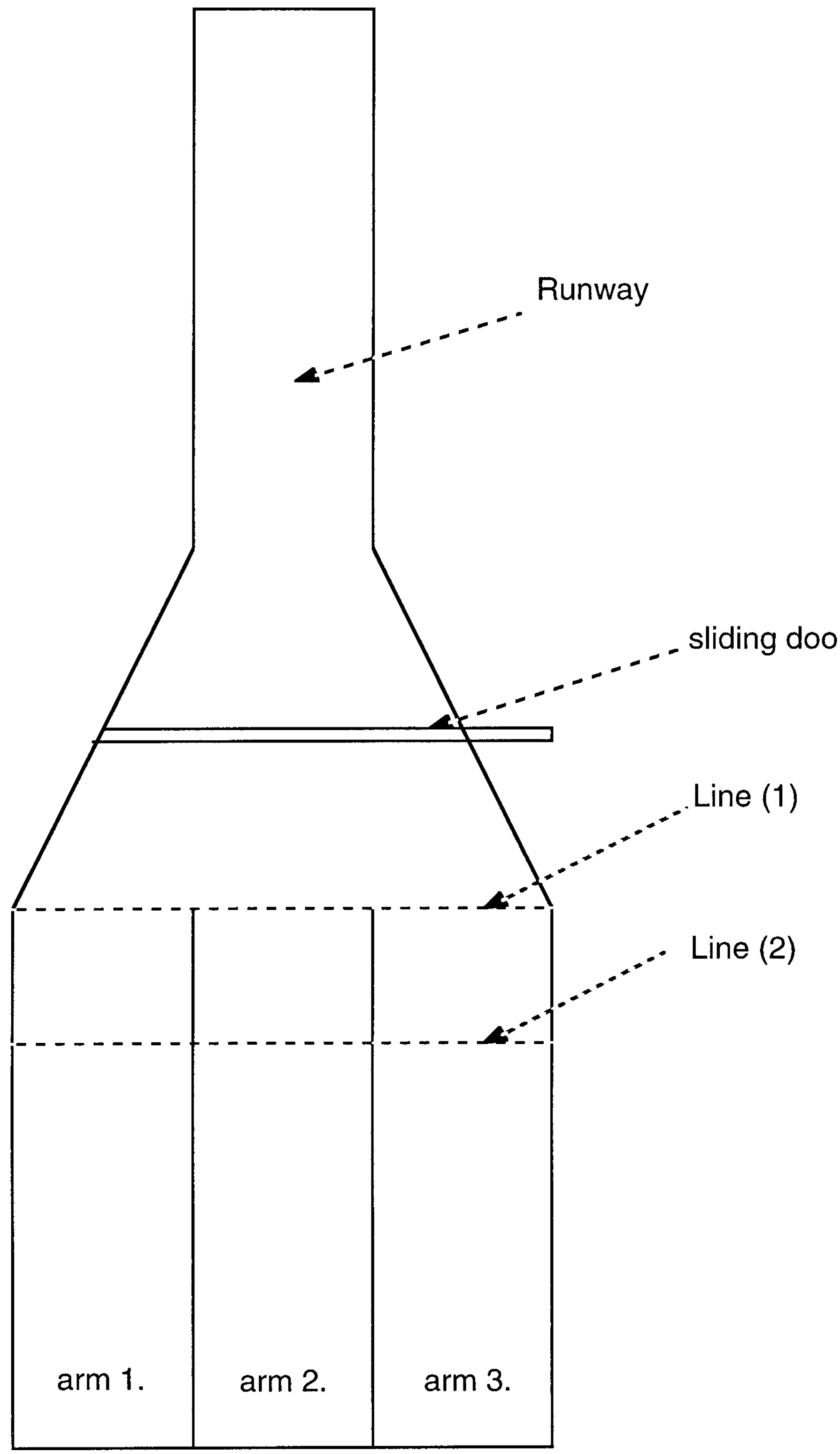
The statistical significance of Merrell's I value was evaluated using a 2x2 contingency table with alpha=0.05. $H_O$: there is no difference in the insemination rates among the based on the number of pairings between: (1) northern males and northern females, (2) northern males and southern females, (3) southern males and southern females and (4) southern males and northern females. This design involved multiple trials for each individual salamander and technically violated the assumption of independent observation of the Chi square test. Houck et al. (1988) suggested that the courtship trials are independent since they involve unique male-female pairs, validating the design.

Experiment 2. Male Olfactory Discrimination

Male olfactory discrimination ability was tested by presenting males with three papers that were produced by using pieces of soiled substrate paper from the maintenance containers of northern and southern females, and a control paper that had not been exposed to female salamanders. These soiled papers have been recognized by other authors as containing chemical cues and in this study will simply be referred as soiled papers or papers (Uzendoski and Verrrell 1993, Anthony 1993). Male salamanders were placed in a three-armed maze that contained the three types of paper. The maze was a modification of one described in Uzendoski and Verrell (1993) which had only two arms. The construction of a third arm allowed the introduction of a blank or control. The maze was constructed from Plexiglas which consisted of a 15 cm runway with three arms, 11cm long. The runway and arms were 2.54 cm wide and the maze height was 2.54 cm (Fig.2)

Two identical mazes labeled A and B were used during these experiments. To minimize any minor differences in construction or other factors, the mazes were washed and alternated between trials. For example, male NM1 (northern population) was tested first in maze A and second in maze B. A particular male salamander was tested not more than once in two days.

If the soiled papers were placed in the same arms for each of the trials the handedness of the salamander might affect the results. The salamander might also give a learned response after being presented with


Runway
sliding door
Line (1)
Line (2)
arm 1.
arm 2.
arm 3.

the same positions several times. These possible problems were minimized by randomly assigning the papers to each arm before each trial was run.

Ten males from each population were used in this experiment. Males were labeled 1 through 10 and were also assigned the letters NM to represent the northern male population and SM to represent the southern male population. Males were tested ten times each, to give a total of 100 trials for each population.

The soiled papers were produced by nine females from each population, since at test time only nine gravid females from each population were available. Northern females (NF) were assigned a number 1-9. The southern females (SF) were similarly assigned numbers 1-9. This reduced the possible confusion between females when administrating tests. The control was always labeled BL.

The protocol for a pheromone discrimination test was as follows. Each maze was washed and dried. In preparation of the test, the runway and one of the arms of the maze were lined with precut filter paper. This paper was then dampened with 3 ml of water from the male's home site. The other two arms were lined with filter paper that had been soiled. These papers were precut and stored in a female's container for three weeks prior to the test date. This time period allowed the papers to become sufficiently soiled with feces and skin secretions. It was assumed that these materials represent species specific olfactory stimuli (Uzendoski and Verrell 1993). The papers were removed from the female's maintenance box and placed directly into the assigned arm of the maze. Each of the precut papers, including the blanks, extended into the runway 0.5 cm. This ensured that the salamander would be presented with the each of the papers.

One paper from each of the female populations was used. The papers selected were the same for each maze. For example, a northern male would be presented with a northern female paper, a southern female paper, and a control. During that trial in a different maze, a southern male would be presented with the same papers. To illustrate, consider trial one for NM1 and SM1. These two male salamanders were presented with the papers in this order from left to right in the arms of the mazes; (NF1, SF1, BL). The northern salamander (NM1) in this trial was placed in maze A, and the southern salamander (SM1) in maze B. Salamander (NM1) was then placed in maze B during its second trial. During the second trial for (NM1 and SM1) the papers were administrated (BL-SF2-NF2)(Appendix A). Care was taken that no two females contributed the same set of papers to a particular salamander. This was done so the male salamander would not give a learned response to repeat papers.

Each maze was covered with a clear lid that had two inscribed lines (Fig. 2) to reduce possible ambiguity in determining the salamander's location in the maze. The salamander was considered to be in an arm of the maze when its jaw angle passed the second line and out of the maze when it passed the first line.

To initiate a trial, each salamander was placed in a maze runway and a five minute acclimation period followed. Salamanders were prevented from entering the arms of the maze by a door(Fig. 2). After the five minute period, the doors of both mazes were simultaneously opened. The arms selected by the salamanders were recorded along with the time spent in an arm. Each test was 25 minutes long. To prevent distractions during the test, a video camera was used to record the data with no human present. The tape was then reviewed with a VCR that had a real time counter.

This experiment was designed assuming that males will either prefer conspecific papers or heterospecific papers and the preference for a particular paper is an indicator of a species preference. Two variables were recorded from this series of olfactory tests, the first arm chosen and the time spent in a type of soiled paper. Statistical analyses were performed to detect any preferences of the papers. $H_O$: there is no difference in the choices made by the two populations of salamanders.

# RESULTS

## Experiment 1. Sexual Compatibility

Courtship success rates did not exceed 50 percent for any of the trial dates. After the first trial date, success rates varied between 20 to 35 percent (Table 2).

Among northern males, as a group, the courtship success rates remained fairly constant, varying between 30 to 60 percent throughout the experiment (Table 3). Eighty percent of individual males within this group were successful in transferring spermatophores to northern females, while seventy percent of northern males were similarly successful with southern females (Table 4).

Southern males, on the other hand, after the first night, were successful ten percent of the time (Table 3). Only thirty percent of the individual southern males were successful in transferring spermatophores to females of either population (Table 4).

The degree of sexual activity among males can be determined by comparing the number of successful transfers with the number of opportunities to do so. Among northern males this ratio varied from zero (0.0%) to 5/6 (83.3%). The total number of spermatophore transfers during the sixty trial nights was 29 (Table 4). In contrast, among southern males, the ratio ranged from zero (0.0%) to 3/6 (50.0%) and with nine spermatophores transferred during the same period (Table 4).

As a group, northern female success rates varied between zero and seventy percent throughout the experiment (Table 3). Ninety percent of individual northern females were successful with northern males and thirty percent with southern males (Table 5). The overall success rate among northern females varied between seventeen and fifty percent (Table 5).

Southern females, as a group, were successful 20 to 40 percent of the time throughout the experiment (Table 3). Individually, eighty percent of southern females picked up spermatophores of northern males and sixty percent of those of southern males.(Table 5) In contrast to the males, the combined success rates were identical at 19 (Table 5).

The results showed that insemination occurred in all four types of pairings (Table 6). In fact, 27% (16 of 60) of interpopulational pairings

**Table 2**. Results of Courtship Encounters for Two Ohio Populations of *Eurycea bislineata,* the Two-lined Salamander.

| Source of Females | Trial night: 12/06/94 | 12/11/94 | 12/16/94 | 12/21/94 | 12/27/95 | 1/04/95 |
|---|---|---|---|---|---|---|
| | Males | | | | | |
| NF1 | NM2* | SM2 | NM 3* | SM3 | NM4 | SM4 |
| NF2 | NM3* | SM3* | NM4* | SM4 | NM5 | SM5 |
| NF3 | NM4* | SM4 | NM5* | SM5 | NM1 | SM1 |
| NF4 | NM5* | SM5 | NM1* | SM1 | NM2 | SM2 |
| NF5 | NM1* | SM1 | NM2* | SM2 | NM3 | SM3 |
| NF'6 | SM'7* | NM'7 | SM'8 | NM'8 | SM'9 | NM'9* |
| NF'7 | SM'8 | NM'8 | SM'9 | NM'9* | SM'10 | NM'10 |
| NF'8 | SM'9* | NM'9 | SM'10 | NM'10 | SM'6 | NM'6 |
| NF'9 | SM'10 | NM'10 | SM'6 | NM'6* | SM'7 | NM'7* |
| NF'10 | SM'6 | NM'6* | SM'7 | NM'7* | SM'8 | NM'8 |
| SF1 | NM'7 | SM'7 | NM'8* | SM'8 | NM'9* | SM'9 |
| SF2 | NM'8 | SM'8 | NM'9 | SM'9 | NM'10 | SM'10* |
| SF3 | NM'9* | SM'9 | NM'10 | SM'10* | NM'6 | SM'6 |
| SF4 | NM'10 | SM'10 | NM'6 | SM'6 | NM'7* | SM'7 |
| SF5 | NM'6 | SM'6 | NM'7 | SM'7 | NM'8* | SM'8 |
| SF'6 | SM2* | NM2* | SM3 | NM3* | SM4 | NM4* |
| SF'7 | SM3 | NM3* | SM4 | NM4* | SM5* | NM5 |
| SF'8 | SM4 | NM4* | SM5* | NM5 | SM1 | NM1* |
| SF'9 | SM5* | NM5 | SM1 | NM1 | SM2 | NM2 |
| SF'10 | SM1 | NM1 | SM2 | NM2* | SM3 | NM3 |
| percent success | 50.0% | 25.0% | 35.0% | 35.0% | 20% | 25% |

NF= northern female population

SF= southern female population

NM= northern male population

SM= southern male population

*= successful insemination

**Table 3.** Courtship Success per Trial Night for Two Ohio Populations of *Eurycea bislineata*, the Two-lined Salamander.

| | night 1 | night 2 | night 3 | night 4 | night 5 | night 6 | Mean |
|---|---|---|---|---|---|---|---|
| NM | 6/10 | 4/10 | 6/10 | 6/10 | 3/10 | 4/10 | 4.8 |
| SM | 4/10 | 1/10 | 1/10 | 1/10 | 1/10 | 1/10 | 1.5 |
| NF | 7/10 | 2/10 | 5/10 | 3/10 | 0/10 | 2/10 | 3.2 |
| SF | 3/10 | 3/10 | 2/10 | 4/10 | 4/10 | 3/10 | 3.2 |

NF= northern female population
SF= southern female population
NM= northern male population
SM= southern male population

**Table 4.** Individual Male Courtship Success Rates for Two Ohio Populations of *Eurycea bislineata,* the Two-lined Salamander.

| Northern Males | Northern Females (Individual) | Southern Females (Individual) | combined success rate (overall) | combined success rate (overall %) |
|---|---|---|---|---|
| NM1 | 2 | 1 | 3/6 | 50.0% |
| NM2 | 2 | 2 | 4/6 | 66.6% |
| NM3 | 2 | 2 | 4/6 | 66.6% |
| NM4 | 2 | 3 | 5/6 | 83.3% |
| NM5 | 2 | 0 | 2/6 | 33.3% |
| NM6 | 2 | 0 | 2/6 | 33.3% |
| NM7 | 2 | 1 | 3/6 | 50.0% |
| NM8 | 0 | 2 | 2/6 | 33.3% |
| NM9 | 2 | 2 | 4/6 | 66.6% |
| NM10 | 0 | 0 | 0/6 | 0.0% |
| TOTALS | 8/10 | 7/10 | 29/60 | |
| TOTALS % | 80% | 70% | 48.33% | |

| Southern Males | Northern Females (Individual) | Southern Females (Individual) | combined success rate (overall) | combined success rate (overall %) |
|---|---|---|---|---|
| SM1 | 0 | 0 | 0/6 | 0.0% |
| SM2 | 0 | 1 | 1/6 | 16.6% |
| SM3 | 1 | 0 | 1/6 | 16.6% |
| SM4 | 0 | 0 | 0/6 | 0.0% |
| SM5 | 0 | 3 | 3/6 | 50.0% |
| SM6 | 0 | 0 | 0/6 | 0.0% |
| SM7 | 1 | 0 | 1/6 | 16.6% |
| SM8 | 0 | 0 | 0/6 | 0.0% |
| SM9 | 1 | 0 | 1/6 | 16.6% |
| SM10 | 0 | 2 | 2/6 | 33.3% |
| TOTALS | 3/10 | 3/10 | 9/60 | |
| TOTALS % | 30.0% | 30.0% | 15.0% | |

NF= northern female population

SF= southern female population

NM= northern male population

SM= southern male population

**Table 5.** Individual Female Courtship Success Rates for Two Ohio Populations of *Eurycea bislineata,* the Two-lined Salamander.

| northern Females | northern males (Individual) | southern males (Individual) | combined success rate (overall) | combined success rate (overall %) |
|---|---|---|---|---|
| NF1 | 2 | 0 | 2/6 | 33.3% |
| NF2 | 2 | 1 | 3/6 | 50.0% |
| NF3 | 2 | 0 | 2/6 | 33.3% |
| NF4 | 2 | 0 | 2/6 | 33.3% |
| NF5 | 2 | 0 | 2/6 | 33.3% |
| NF6 | 1 | 1 | 2/6 | 33.3% |
| NF7 | 1 | 0 | 1/6 | 16.6% |
| NF8 | 0 | 1 | 1/6 | 16.6% |
| NF9 | 2 | 0 | 2/6 | 33.3% |
| NF10 | 2 | 0 | 2/6 | 33.3% |
| TOTALS | 9/10 | 3/10 | 19/60 | |
| TOTALS % | 90.0% | 30.0% | 31.6% | |

| southern Females | northern males (Individual) | southern males (Individual) | combined success rate (overall) | combined success rate (overall %) |
|---|---|---|---|---|
| SF1 | 2 | 0 | 2/6 | 33.3% |
| SF2 | 0 | 1 | 1/6 | 16.6% |
| SF3 | 1 | 1 | 2/6 | 33.3% |
| SF4 | 1 | 0 | 1/6 | 16.6% |
| SF5 | 1 | 0 | 1/6 | 16.6% |
| SF6 | 3 | 1 | 4/6 | 66.6% |
| SF7 | 2 | 1 | 3/6 | 50.0% |
| SF8 | 2 | 1 | 3/6 | 50.0% |
| SF9 | 0 | 1 | 1/6 | 16.6% |
| SF10 | 1 | 0 | 1/6 | 16.6% |
| TOTALS | 8/10 | 6/10 | 19/60 | |
| TOTALS % | 80% | 60% | 31.6% | |

NF= northern female population

SF= southern female population

**Table 6.** Individual First Choices of Males in Northern (NM) and Southern Ohio Populations (SM) of *Eurycea bislineata,* the Two-Lined Salamander.

| | Males' first choice. | | | | Males' first choice. | | |
|---|---|---|---|---|---|---|---|
| Northern Males | Northern Females | Southern Females | Control | Southern Males | Northern Females | Southern Females | Control |
| NM1 | 6 | 4 | 0 | SM1 | 4 | 4 | 2 |
| NM2 | 5 | 5 | 0 | SM2 | 6 | 4 | 0 |
| NM3 | 5 | 5 | 0 | SM3 | 7 | 3 | 0 |
| NM4 | 3 | 6 | 1 | SM4 | 6 | 4 | 0 |
| NM5 | 5 | 5 | 0 | SM5 | 3 | 4 | 3 |
| NM6 | 5 | 3 | 2 | SM6 | 3 | 7 | 0 |
| NM7 | 7 | 3 | 0 | SM7 | 2 | 5 | 3 |
| NM8 | 5 | 5 | 0 | SM8 | 5 | 5 | 0 |
| NM9 | 7 | 3 | 0 | SM9 | 6 | 4 | 0 |
| NM10 | 3 | 5 | 2 | SM10 | 4 | 5 | 1 |
| TOTAL% | 51% | 44% | 5% | | 46% | 45% | 9% |

were successful and 37% (22 of 60) of the intrapopulational pairings were successful. Merrell's isolation value was .727 and the Chi square value of 1.31 (critical value of 3.841 (.25<P< .50) alpha .05) was not significant.

Experiment 2. Male Olfactory Discrimination.

During a trial run, a northern and southern male were tested simultaneously (Table 6). Each salamander had the potential to make ten first choices since there were ten trials. For example NM1 chose a northern paper six times and a southern paper four times. A few of the salamanders never made a choice and therefore had less than ten choices, for example SM1.

These data were analyzed to detect the male's ability to choose conspecific or heterospecific female soiled substrates. In the first analysis the Wilcoxon matched-pairs signed-ranks test was used to detect those possible preferences. Alpha was set at 05. This test was considered two-tailed since no prediction was made prior to testing about the discrimination ability of the males for the soiled papers. $H_O$: there is no difference in the first choices made by male salamanders of either population. Neither northern nor southern males showed a significant preference for a particular type of paper (.1>P>.25 and P>.25, respectively).

The second analysis compared trial partner's first choices, since the partners were presented with the same papers in identical order simultaneously throughout the experiment, e.g., did NM1 and SM1 make the same first choices during the ten trials? If the partners made the same choice, this was recorded in column one, and if they made a different choice, in column two (Table 7). A two tailed Wilcoxon matched-pairs signed-ranks test of first choices, $H_O$: there is no difference in trial partner's first choices, was not significant (P> .20), alpha= 0.05.

The time spent by salamanders in a particular type of soiled paper was recorded throughout the entire 250 minute test period (ten trials x 25 min. each). A two-tailed Wilcoxon matched-pairs signed-ranks test (alpha=.05), carried out on the combined data (Table 8) to test $H_O$: there is no difference in time spent in a particular type of soiled substrate for a

**Table 7.** Trial Partners First Choices for Two Ohio Male Populations of *Eurycea bislineata,* the Two-lined Salamander.

| | First choices | |
|---|---|---|
| | Same | Different |
| NM1-SM1 | 4/10 | 5/10 |
| NM2-SM2 | 4/10 | 6/10 |
| NM3-SM3 | 4/10 | 6/10 |
| NM4-SM4 | 3/10 | 7/10 |
| NM5-SM5 | 3/10 | 7/10 |
| NM6-SM6 | 3/10 | 7/10 |
| NM7-SM7 | 4/10 | 6/10 |
| NM8-SM8 | 6/10 | 4/10 |
| NM9-SM9 | 5/10 | 5/10 |
| NM10-SM10 | 6/10 | 4/10 |

This table shows the number of times the partners chose the "same" or "different" paper for their first choice. For example NM1 and SM1 first choices were the same four times and different five times. The two columns may not add up to ten because a choice may not have been made during a trial.

NM= northern male

SM= southern male

Ratios represent actual number of choices/ possible chances.

**Table 8.** Total Time Spent in a Particular Type of Soiled Substrate by Two Ohio Male Populations of *Eurycea bislineata,* the Two-lined Salamander.

| Northern Males | Minutes in a Paper. Northern Female Paper | Southern Female Paper |
|---|---|---|
| NM1 | 35/250 | 26/250 |
| NM2 | 40/250 | 30/250 |
| NM3 | 28/250 | 42/250 |
| NM4 | 51/250 | 32/250 |
| NM5 | 53/250 | 51/250 |
| NM6 | 15/250 | 18/250 |
| NM7 | 27/250 | 18/250 |
| NM8 | 12/250 | 19/250 |
| NM9 | 41/250 | 24/250 |
| NM10 | 36/250 | 50/250 |
| TOTAL | 338/2500 | 310/2500 |

| Southern Males | Minutes in a Paper Northern Female Paper | Southern Female Paper |
|---|---|---|
| SM1 | 21/250 | 39/250 |
| SM2 | 35/250 | 20/250 |
| SM3 | 36/250 | 45/250 |
| SM4 | 54/250 | 44/250 |
| SM5 | 14/250 | 21/250 |
| SM6 | 25/250 | 31/250 |
| SM7 | 16/250 | 44/250 |
| SM8 | 46/250 | 32/250 |
| SM9 | 31/250 | 35/250 |
| SM10 | 34/250 | 34/250 |
| TOTAL | 312/2500 | 345/2500 |

NM=northern male

SM= southern male

Ratios represent: time in a particular soiled substrate / (250) total minutes possible given population of males, was not significant ($P > .25$) for either population.

# DISCUSSION

A critical factor affecting reproductive isolation among sexually reproducing species has been natural selection for isolating mechanisms. These essential mechanisms may occur prior to fertilization and are then known as premating mechanisms or after fertilization and then are called postmating mechanisms. A basic premating isolation mechanisms is the ability to recognize available members of the opposite sex among conspecific animals. Among salamanders, chemical recognition, i.e., the ability to distinguish individual body chemicals, fecal material, etc., appears to be a vital part of pre- courtship behavior, forming part of a complex suite of isolating mechanisms (Anthony 1993). Divergence of socially significant chemical cues has been implicated as an important factor in the maintenance of sexual incompatibility among conspecific populations (Ovaska 1989). Such chemical cues are an important component of the mate recognition systems of plethodontid salamanders, and like courtship behavior, can diverge rapidly (Uzendoski and Verrell 1993). The evidence presented by these previous investigators suggested that chemical cues may be important in other plethodontids.

Studies of territoriality in plethodontids, show definite preferences either for or against conspecific cues. Jaeger and Gergitis (1979) using *Plethodon cinereus* showed that conspecific cues repelled salamanders. Anthony (1993) found that *P. caddoensis* and *P. ouachitae* preferred conspecific cues, possibly to find a mate. Uzendoski and Verrell (1993) using *Desmognathus ochrophaeus and D. fuscus* performed an actual preference test similar to the present one. They found that two populations of male salamanders could discriminate between conspecific and heterospecific females on the basis of differences in substrate-born chemical cues. Both populations of males were more likely to approach conspecific female cues.

Results of the present study showed that preferences did not exist among the northern and southern populations of *Eurycea bislineata.* Analyses of male olfactory discrimination experiments showed no such preferences for either conspecific or heterospecific papers. The results of the olfactory discrimination experiments support the null hypothesis that these two populations represent a single species of salamander.

Furthermore, results of the sexual compatibility tests reinforce this hypothesis. During the current study of *Eurycea bislineata,* Merrell's isolation value was I=.727 which indicates that random mating occurred. This suggests compatibility exists between these two populations of *Eurycea bislineata*. One would not expect two populations that are reproductively isolated to exhibit a 27% interpopulational insemination rate. Interspecific matings are costly and such a high rate would reduce the fitness of the salamanders involved. Uzendoski and Verrell (1993) in a study of *D. ochrophaeus*. and *D. fuscus*, showed a 53% intraspecific success rate and a 0.0 % interspecific success rate. In contrast, to the present study their results clearly showed incompatibility between these two species. Houck et al. (1988) reported a 50% success rate for intraspecific crossings and 0.0% for interspecific crossings between *D. ochrophaeus* and *D. fuscus*. In both of these studies, the isolation value (I) is zero, indicating that high incompatibility is consistent with reproductive isolation.

Plethodontids show seasonal variability in their insemination rates. Houck et al.(1985) found that insemination rates were higher in spring than in fall in a North Carolina population of *D. ochrophaeus*. These rates varied from 20-80% throughout the seasons. Houck et al. (1988) performed an experiment in winter with two populations of *D. ochrophaeus* from Tennessee that were 134 km. apart and found rates that were intermediate to those found in the previous study. They found an insemination success rate of 61% for intrapopulational pairings and 27% for interpopulational pairings as opposed to the current study with insemination rates of 37% in intrapopulational pairings and 27% for interpopulational pairings. These rates suggest that the *D. ochrophaeus* salamanders may have been more sexually active compared to *Eurycea bislineata* in the current study. Verrell (1988a) found an intrapopulational success rate of 39% in *D. ochrophaeus*. Verrell explained that the differences he observed were seasonal. Insemination rates obtained in the present study are not unreasonable because they fall within the range of variability reported for other plethodontids.

Insemination success rates per trial night were consistent throughout the duration of the experiment, varying between 5 and 10 inseminations, indicating that the salamanders were sexually active throughout the 29 days. The results of the present study were consistent for northern and southern females with final insemination rates of 32.% (19/60 encounters, Table 5). On the other hand, males exhibited different success rates. Northern males inseminated females during 48% of the courtships (29/60 encounters, Table 5), while southern males were successful during 15% of the courtships (9/60 encounters, Table 5). Clearly the southern males were not as sexually active.

It is difficult to explain this difference between the males of the two populations. A possible explanation is that southern males experienced a lull in seasonal sexual activity. Houck et al. (1985) discovered that salamander insemination rates among members of the species *D. ochrophaeus*, change throughout the year. The present results may not have occurred during the southern male's peak of sexual activity accounting for the low rate.

Guttman and Karlin (1986) suggested that certain populations of two-lined salamanders in Ohio represented a pair of cryptic species. Their theory was based on electrophoretic data gathered during an enzymatic study of the *Eurycea bislineata* complex. In the current study, tests of neither sexual compatibility nor male olfactory discrimination between the northern and southern populations support this theory. Current evidence suggests that these two Ohio populations of *Eurycea bislineata,* the two-lined salamander, represent a single species.

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# APPENDIX A.

**Appendix A.** Trial Sequences for Olfactory Discrimination Test.

| | Trial number | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Male # | 6 | 7 | 8 | 9 | 10 |
| NM1-SM1 | NF1-SF1-BL | BL-SF7-NF7 | BL-SF2-NF2 | SF3-BL-NF3 | NF4-BL-SF4 |
| | SF5-NF5-BL | BL-NF6-SF6 | NF8-BL-SF8 | SF9-NF9-BL | SF1-BL-NF9 |
| NM2-SM2 | SF2-BL-NF1 | SF1-NF2-BL | NF3-BL-SF4 | BL-SF3-NF4 | BL-NF5-SF6 |
| | SF5-NF6-BL | NF7-BL-SF8 | SF7-BL-NF8 | BL-SF2-NF9 | NF8-SF9-BL |
| NM3-SM3 | BL-SF4-NF2 | NF1-SF3-BL | SF7-NF5-BL | SF1-BL-NF3 | NF4-BL-SF2 |
| | BL-NF6-SF8 | BL-SF5-NF7 | NF8-BL-SF6 | SF3-NF9-BL | SF9-BL-NF7 |
| NM4-SM4 | NF3-BL-SF8 | BL-SF7-NF4 | BL-NF-SF6 | SF5-NF6-BL | NF7-BL-SF4 |
| | SF3-BL-NF8 | BL-SF2-NF9 | NF6-SF2-BL | SF4-BL-NF1 | SF9-NF2-BL |
| NM5-SM5 | NF4-BL-SF8 | SF5-NF5-BL | SF7-BL-NF3 | BL-NF6-SF6 | BL-SF3-NF7 |
| | NF8-BL-SF4 | SF1-NF9-BL | SF2-BL-NF5 | NF1-SF9-BL | BL-SF5-NF2 |
| NM6-SM6 | BL-NF5-SF8 | BL-SF5-NF4 | NF3-BL-SF6 | SF2-NF6-BL | NF7-BL-SF1 |
| | SF7-BL-NF8 | BL-SF3-NF9 | NF4-SF6-BL | SF6-BL-NF1 | SF9-NF2-BL |
| NM7-SM7 | BL-SF3-NF7 | NF8-BL-SF2 | SF5-NF9-BL | SF7-BL-NF3 | NF1-SF8-BL |
| | BL-SF1-NF2 | SF4-BL-NF3 | NF4-BL-SF6 | SF7-NF5-BL | BL-NF6-SF9 |
| NM8-SM8 | SF3-NF6-BL | NF7-BL-SF2 | SF8-BL-NF8 | BL-SF8-NF9 | NF2-SF5-BL |
| | SF1-BL-NF1 | SF4-NF2-BL | NF3-BL-SF1 | BL-SF6-NF4 | BL-NF5-SF7 |
| NM9-SM9 | BL-SF4-NF7 | BL-NF6-SF9 | NF8-BL-SF2 | SF1-NF9-BL | SF8-BL-NF1 |
| | NF1-SF7-BL | BL-SF3-NF2 | SF9-BL-NF3 | NF4-BL-SF6 | SF5-NF5-BL |
| NM10-SM10 | NF7-BL-SF6 | SF9-BL-NF8 | BL-SF8-NF9 | NF7-SF3-BL | SF4-BL-NF1 |
| | SF5-NF2-BL | NF3-BL-SF2 | BL-SF1-NF4 | BL-NF5-SF3 | SF7-NF6-BL |

NM=northern male

SM=southern male

NF=northern female

SF= southern female

BL= control