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Source: Copeia, 105(2) : 261-268

Published By: The American Society of Ichthyologists and Herpetologists

URL: <https://doi.org/10.1643/CE-16-565>

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Sexual Isolation between Two Sympatric *Desmognathus* in the Gulf Coastal Plain

Jennifer Y. Lamb¹

A substantial body of work exists examining courtship in lungless salamanders (family Plethodontidae), but there are many species and population pairs that have not been tested for sexual isolation. Spotted (*Desmognathus conanti sensu lato* [SL]) and Southern Dusky Salamanders (*D. aff. auriculatus*) occur sympatrically within drainages in South Mississippi. I crossed individuals from three populations of *D. aff. auriculatus* ($n = 6$ individuals) and seven populations of *D. conanti* (SL) ($n = 10$) occurring in the Pascagoula and Pearl River basins. I alternated homospecific ($n = 23$) and heterospecific ($n = 23$) trials across nights in the spring and summer of 2014 and 2015, and I used GoPro™ HERO3 cameras and time-lapse photography to record the behaviors of a random subset of these pairs, as well as to determine the stage of courtship reached. Seventeen of the 23 homospecific trials resulted in spermatophore deposition and 14 in insemination. None of the 23 heterospecific trials (*D. aff. auriculatus* ♂ x *D. conanti* [SL] ♀; or *D. conanti* [SL] ♂ x *D. aff. auriculatus* ♀) reached this stage of courtship, but ten of the 14 photographed, heterospecific pairs exhibited pursuit and/or persuasive behaviors (i.e., pheromone transfer). These results suggest that *D. aff. auriculatus* and *D. conanti* (SL) are completely sexually isolated and that isolation is likely chemically mediated. I used a total of eight polymorphic microsatellite loci to genotype offspring from two clutches oviposited by a single female *D. aff. auriculatus* and one clutch oviposited by a female *D. conanti* (SL). Strict exclusion suggested that each clutch was sired by a single male, and that female *D. aff. auriculatus* and female *D. conanti* (SL) were capable of storing sperm for at least 4 mo 5 days and 3 mo 3 days, respectively. The data presented here further our understanding of the reproductive ecologies of *Desmognathus* in the historically understudied Gulf Coastal Plain.

REPRODUCTIVE isolation—one line of evidence that can be used in species delineation (de Queiroz, 2005)—occurs when populations are unable to successfully interbreed and create fertile offspring. This phenomenon can occur prior to (i.e., pre-zygotic) or after (i.e., post-zygotic) a sperm fertilizes an egg cell (Coyne and Orr, 2004). There are many examples of incomplete reproductive isolation among Caudata (Voss and Shaffer, 1996; Mead and Verrell, 2002; Tilley, 2016), and there has been an impressive amount of work investigating sexual incompatibility among the woodland (genus *Plethodon*) and dusky (genus *Desmognathus*) salamanders (family Plethodontidae; Arnold et al., 1993; Mabry and Verrell, 2004). Sexual incompatibility, also known as sexual isolation (Dobzhansky, 1935), is a type of pre-zygotic reproductive isolation encompassing scenarios in which the act of insemination does not occur (Dobzhansky, 1935; Arnold et al., 1993; Mabry and Verrell, 2004). The family Plethodontidae is an interesting group with which to examine a variety of hypotheses pertaining to sexual isolation. This family boasts of a large number of species that are distributed both allopatrically and sympatrically (Lannoo, 2005), and many species can easily be maintained and will readily court in the laboratory (Arnold et al., 1993). Courtship behaviors appear to be remarkably similar across congeneric taxa (Verrell, 1999); therefore, we can ask whether sexual isolation is maintained by behavioral and/or chemical cues (Arnold et al., 1993). Finally, because the degree of reproductive isolation between not only different species, but also among conspecific populations, varies with geographic and genetic distance (Mabry and Verrell, 2004; Tilley, 2016) there are opportunities to examine the role of both distances in the evolution and maintenance of sexual isolation.

Species that are broadly sympatric may exhibit a greater degree of sexual isolation than those with narrow overlap along the peripheral portions of their ranges. Complete sexual isolation has been observed between multiple pairs of sympatric species of plethodontids, but pre- and post-zygotic

barriers can be leaky (Mabry and Verrell, 2004; Tilley, 2016). For example, *in situ* hybridization has been documented between Northern (*D. fuscus*) and Allegheny Mountain Dusky (*D. ochrophaeus*) salamanders (Karlin and Guttman, 1981; Sharbel et al., 1995). Fewer studies examining sexual isolation have been undertaken for taxa which occur in the Gulf Coastal Plain (Verrell, 1990a; Kozak, 2003; Mabry and Verrell, 2004).

Both the Southern Dusky (*D. auriculatus*; Holbrook, 1838) and the Spotted Dusky (*D. conanti*; Rossman, 1958) salamanders have widely overlapping ranges within the Gulf Coastal Plain (Means, 2005; Means and Bonett, 2005). Populations attributed to *D. auriculatus* in Mississippi and Louisiana are genetically (Lamb, 2016) and morphologically distinct from topotypic *D. auriculatus* in Georgia and Florida, and proposed taxonomic revisions for this historically confounding group are pending (Means et al., in press). Hereafter I refer to these populations of salamanders in Mississippi and Louisiana as *D. aff. auriculatus*. *Desmognathus conanti* also includes multiple, divergent genetic lineages (Karlin and Guttman, 1986; Bonett, 2002; Kozak et al., 2005; Beamer and Lamb, 2008; Lamb, 2016), but their taxonomic status is uncertain. Hereafter populations of this species are referred to as *D. conanti sensu lato* (SL).

Desmognathus aff. auriculatus and *D. conanti* (SL) have the opportunity to interact *in situ*. These species occur within the same tributaries in the Pearl and Pascagoula rivers in South Mississippi, and they can occasionally be found in the same microhabitats at lotic sites (Valentine, 1963; Means et al., in press). Individuals, especially older males (Valentine, 1963), are grossly phenotypically similar and reproductive adults overlap in body size (e.g., male *D. aff. auriculatus* and *D. conanti* [SL] mean snout-to-vent lengths [SVL] are 56 ± 6 mm and 49 ± 4 mm, respectively; female mean SVLs are 53 ± 5 mm and 48 ± 9 mm, respectively; Means et al., in press). Our limited knowledge of the reproductive phenologies of both species in this part of their ranges suggests that their breeding seasons partially coincide. For example, I encountered a

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Submitted: 15 December 2016. Accepted: 23 February 2017. Associate Editor: C. Beachy.

© 2017 by the American Society of Ichthyologists and Herpetologists DOI: 10.1643/CE-16-565 Published online: 22 June 2017

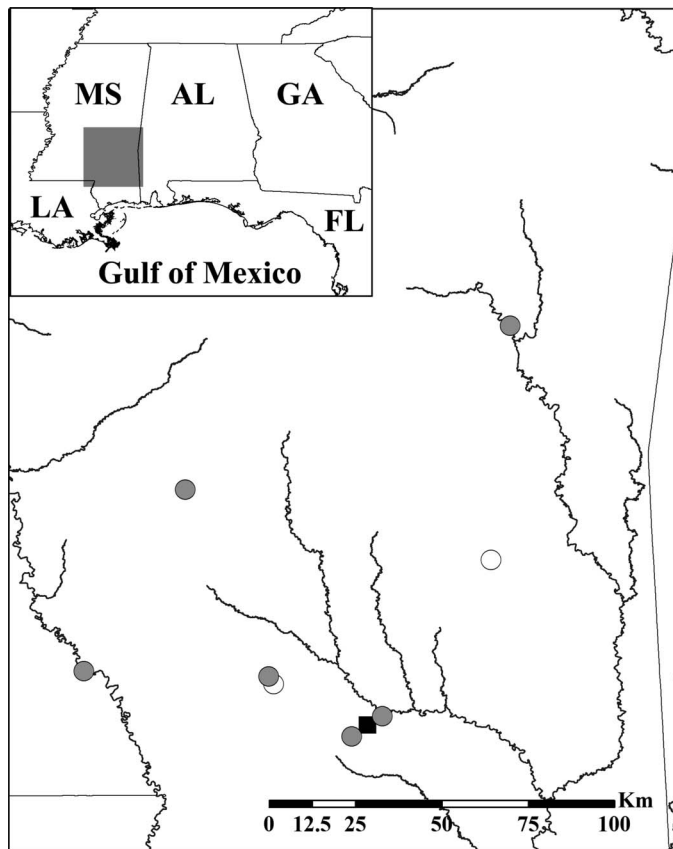


Fig. 1. Collection localities in the Pascagoula and Pearl river drainages. The inset includes a portion of the Gulf Coastal Plain with state names abbreviated and the study area enclosed within a gray square. White and gray circles mark locations from which only *D. aff. auriculatus* or *D. conanti* (SL) were collected, respectively. The black square marks a locality from which both species were collected syntopically.

female *D. conanti* (SL) from a Pascagoula population (Jones Co.) with a sperm cap in her cloaca in May, and I have observed both of these species tending eggs in the late summer (i.e., *D. conanti* [SL] from a Pearl River population, Marion Co.), and eggs or very recent hatchlings in the fall (i.e., *D. conanti* [SL] from Jackson Co. and *D. aff. auriculatus* from Forrest Co. in October; unpubl. data). Males with prominent mental glands can be found throughout most of the year for both taxa (unpubl. data). Histological studies of populations of *D. conanti* (SL) elsewhere in the Gulf Coastal Plain suggest that these glands do not fully atrophy and that males may be capable of breeding throughout the year (Rupp, 2016). Given the capacity for female plethodontids to store sperm for anywhere from two to greater than nine months (Marynick, 1971; Sever, 2003; Eddy et al., 2015) as well as the prolonged breeding season in some species, it is feasible that receptive adults of both *D. aff. auriculatus* and *D. conanti* (SL) co-occur spatially and temporally.

I completed multiple crosses in captivity of *D. aff. auriculatus* and *D. conanti* (SL) from several populations in South Mississippi to test whether these salamanders were sexually isolated within the sympatric portions of their ranges. I used GoPro HERO3 cameras (GoPro, Inc., San Mateo, CA) and time-lapse photography to identify the stage of courtship reached as well as to compare behaviors between species. Upon the completion of courtship trials, I allowed females to oviposit naturally and then used multiple microsatellite loci (Lamb et al., 2015) to genotype any

resulting offspring. This allowed me to identify the successful sire(s) for each clutch and to determine the minimum period of time over which females were capable of storing viable sperm.

MATERIALS AND METHODS

Collection and maintenance of animals.—*Desmognathus* aff. *auriculatus* and *D. conanti* (SL) can be difficult to distinguish *in situ*; however, there are discrete phenotypic differences that genetic studies have confirmed are reliable (Means et al., in press). Between 3 April 2014 and 2 February 2015 I collected six *D. aff. auriculatus* and ten *D. conanti* (SL) from nine localities in the Pascagoula and Pearl river drainages in South Mississippi (Fig. 1). I measured snout-to-vent lengths (SVL) and sexed salamanders in the field using the presence of an obvious mental gland to identify males (SVL three *D. aff. auriculatus*: 55 mm, range = 48–63 mm; SVL six *D. conanti* [SL]: 51 mm, range = 42–59 mm) and ovarian follicles visible through the ventral skin to identify females (SVL three *D. aff. auriculatus*: 60 mm, range = 58–62 mm; SVL four *D. conanti* [SL]: 51 mm, range = 47–55 mm). I transported salamanders in individual, re-sealable plastic bags to laboratory facilities at the University of Southern Mississippi where I maintained each in separate, lidded plastic containers (21 × 15 × 10 cm) containing moist paper towels. I kept containers in an environmental chamber set to a 12 hr day:night cycle and constant 20°C. This temperature was within the range of what these species would experience in small streams during the summer months *in situ* (unpubl. data). Once or twice per week, I changed paper towels and fed each salamander between five and seven small crickets. I occasionally supplemented their diets with fruit flies (*Drosophila* spp.) and calcium dusted crickets.

Courtship encounters.—I staged courtship encounters from June–September 2014 and April–May 2015. Encounters took place during the nocturnal cycle, from 2000–0600 h, in lidded plastic shoe boxes (35 × 18 × 12 cm) lined with moist paper towels and under Mood-lites® Passion Red bulbs. I obscured the view between adjacent boxes by wrapping each box in brown paper towels. Homospecific encounters ($n = 23$ total) always preceded heterospecific encounters ($n = 23$ total), and individuals were typically given three nights between each encounter to recuperate (range = 2–5 nights; Verrell, 1988a, 1988b; Arnold et al., 1993). All but one pairing of salamanders was unique. A single male and female *D. aff. auriculatus* were paired in both 2014 and 2015 in order to ensure that each male met a homospecific prior to a heterospecific female. I allowed each female to acclimate for 30 min in the shoe box before adding a single male and beginning the trial. On the following morning, I visually inspected the paper towel and the bottom of the enclosure for remnants of spermatophores. I also inspected the cloaca of each female for the presence of a sperm cap, which can remain visible for ca. 24 hours post-insemination (Verrell, 1991).

GoPro HERO cameras have recently been used in behavioral studies with amphibians (Pašukonis et al., 2014; Greene et al., 2016; Lanctôt et al., 2016) and reptiles (Holding et al., 2015). The small size of these cameras and their ability to capture high resolution, wide-angle photos makes them well suited for documenting behavior in small, dimly lit arenas. I used GoPro HERO3 cameras and 30-second time-lapse photography (photo resolution = 1080 p) to record the full

Table 1. Modified behavioral ethogram used to score time-lapse photography.

Behavior	Description
Head Rub	The head of one salamander remains in contact with some part of the head of the other in at least two sequential photos.
Body Contact	The head of one salamander is in contact with the other's body in one or more sequential photos.
C-Position	The female's chin is on the base of the male's tail and her forelimbs are astride his tail. The male's head and forebody are curved backward and his chin is in contact with the female's head or body. There is no sequential circular motion across two or more photos.
Waltz	The female's chin is on the dorsal surface of the base of the male's tail and the male's head and forebody are curved backward toward the female's neck or dorsum so that he is in a C-shaped position. In two or more sequential photos the pair maintains this orientation and moves in a circular fashion.
Tail-Straddle Walk	In a series of photos, the male slides his body under the head of the female until her chin is positioned on his tail base and her forelimbs straddle his tail. The male then moves forward in a straight line, lateral tail undulation may or may not be apparent, and the female follows, remaining astride his tail.
Gape	The mouth is open in one or more sequential photos.
Bite	One salamander bites the other.

duration of 12 homospecific (seven *D. aff. auriculatus*; five *D. conanti*) and 14 heterospecific (six *D. aff. auriculatus* male x *D. conanti* [SL] female; eight *D. conanti* [SL] male x *D. aff. auriculatus* female) encounters. I chose the couples randomly, and I photographed each from directly above the shoe box via a small hole cut out of the center of the lid. I used the behavioral scale in Verrell and Arnold (1989) to identify the stage of courtship reached by each couple (i.e., 0. no interest; 1. pursuit; 2. persuasion; 3. sperm transfer) with specific behaviors scored using a modified ethogram (Table 1) informed by descriptions in Verrell (1995, 1997), Mead and Verrell (2002), and Plenderleith and Forester (2011). Since 30-second time-lapse photography cannot capture rapid behaviors with short durations such as “jerk,” “nudge,” or “butterfly” (Verrell, 1995, 1997), a comparison of the complete suite of courtship behaviors exhibited by these populations and species is beyond the scope of the present study and the ethogram presented is limited.

Oviposition and tissue collection.—Each summer, after the conclusion of all courtship trials, I placed ovigerous females in individual shoe boxes designed to mimic nesting microhabitats and encourage oviposition. I maintained nesting enclosures under the same light and temperature regime as previously described. Each enclosure contained one or two cover items (i.e., pieces of wood or flat rocks) and clumps of moss (*Sphagnum* sp.) atop a substrate of approximately 1–2 cm of washed sand. I checked shoe boxes for clutches of eggs and provided females with a few small crickets no more frequently than once per week. I added small amounts of water or misted enclosures during these brief disturbances to keep the substrate very moist. When I discovered a clutch, I photographed it as found so that I could estimate the minimum number of eggs produced. I also photographed clutches opportunistically throughout their development. In most cases I allowed females to remain with their clutches until hatching. I had previously collected tissue samples from each adult for other studies. I collected tissue from offspring either by snipping the tail tips of hatchlings or by preserving eggs whole in 100% ethanol. I euthanized hatchlings and adults not involved in other studies with a 20% benzocaine solution (Brown et al., 2004) and preserved them in a 10% formalin solution. Adults were transferred to a 70% solution of ethyl alcohol and ultimately deposited at the Mississippi Museum of Natural Science to serve as locality vouchers.

Paternal exclusion.—I used the conditions specified in Lamb et al. (2015) to amplify six polymorphic microsatellite loci per species (*D. aff. auriculatus*: *Dcon05*, *Dcon14*, *Dcon16*, *Dcon26*, *Dcon34*, and *Dcon36*; *D. conanti* [SL]: *Dcon12*, *Dcon14*, *Dcon26*, *Dcon34*, *Dcon36*, and *Dcon40*) to genotype offspring, known mothers, and any males that had successfully inseminated those females during courtship trials. When individuals or loci amplified inconsistently, I adjusted PCR conditions to include a greater concentration of template (1.5–2 μ L per reaction) and *Taq* (1.5X) and/or I added bovine serum albumin (1–2X). I visualized genotypes on a polyacrylamide gel using a LICOR 4300 DNA analyzer, and I sized alleles using GeneProfiler ver. 4.05 (LICOR Co.).

Multiple paternity has been documented in other *Desmognathus* (Tilley and Hausman, 1976; Houck et al., 1985; Adams et al., 2005), and clutches produced in this study could have been sired by more than one male during trials, or by males that a female encountered in the field prior to being collected. I was able to use complete exclusion to determine which captive male(s) were or were not the father(s) because the females that oviposited were only inseminated by a small number of males in captivity, and because the microsatellites being used were highly polymorphic (Lamb et al., 2015). Complete or strict exclusion is a conservative method of assigning paternity in which a male cannot be the father of a particular offspring if his genotype mismatches that individual at a single locus (Tennessen and Zamudio, 2003). Offspring with alleles not shared by any of the captive potential fathers are assumed to have been the result of sperm stored by the female after successful courtship in the field. This method of paternity analysis assumes that there has been no mutation or genotyping error. To avoid the latter issue, I based each individual's allele scores on the results from at least two successful, independent amplification attempts. I used GenAIEx 6.5 (Peakall and Smouse, 2012) to calculate allelic diversity and other summary statistics for each clutch.

RESULTS

Courtship encounters.—Insemination occurred in 54% of homospecific encounters involving *D. aff. auriculatus* and in 67% of homospecific encounters involving *D. conanti* (SL), and nearly all encounters between *D. conanti* (SL) resulted in the deposition of at least one spermatophore by the male (Table 2). In most encounters a male deposited a single

Table 2. Proportion of encounters in which insemination and spermatophore deposition occurred. Six *D. aff. auriculatus* (three males and three females) and ten *D. conanti* (SL) (six males and four females) were used. The number of inseminations is given first and the number of encounters in which spermatophores were deposited is bracketed. Parentheses enclose the number of encounters of that type (23 homospecific and 23 heterospecific trials in total).

	Male <i>D. aff. auriculatus</i>	Male <i>D. conanti</i> (SL)
Female <i>Desmognathus aff. auriculatus</i>	6 [6] (<i>n</i> = 11)	0 [0] (<i>n</i> = 12)
Female <i>D. conanti</i> (SL)	0 [0] (<i>n</i> = 11)	8 [11] (<i>n</i> = 12)

spermatophore. However, two males, one of each species, deposited two spermatophores during an encounter, and a different male *D. conanti* (SL) deposited three spermatophores in one night.

In homospecific encounters, *D. aff. auriculatus* and *D. conanti* (SL) exhibited similar behaviors, but they differed in 1) the proportion of couples across which behaviors were observed (Table 3), 2) the frequency of occurrence for certain behaviors within individual encounters, and 3) the duration of behaviors. There is insufficient power to complete statistical significance tests due to the small sample sizes used herein, but it is still useful to compare the behaviors observed across encounter types. The C-Position and Waltz (see Table 1), both of which occur during the persuasion stage (Stage 2), varied most notably between taxa. The C-Position may be a precursor to the “snap” (Mead and Verrell, 2002) or the Waltz (Verrell, 1997; pers. obs.). I observed this behavior in a larger proportion of encounters between *D. conanti* (SL) than *D. aff. auriculatus* (Table 3). Of those encounters in which I observed the C-Position, it was performed more frequently per encounter by male *D. conanti* (SL) (mean = 7.8 times per encounter, range = 1–16 times) than by male *D. aff. auriculatus* (mean = 4.3 times per encounter, range = 1–13 times). Further, on multiple occasions and in several encounters, I observed male *D. conanti* (SL) performing the C-Position across sequential photos but alternating the side to which they arched backwards, giving them the appearance of swinging from side to side as they led a female slowly forwards. This swinging C-Position sequence was not observed in *D. aff. auriculatus*. A larger proportion of couples of *D. aff. auriculatus* exhibited the Waltz behavior (Table 3). When the Waltz occurred, male *D. aff. auriculatus* led females for slightly longer periods of time (mean = 2.5 min, range = 1–7 min) and initiated the waltz more frequently per

encounter (mean = 12.0 times, range = 4–29 times) than did *D. conanti*. Only a single male *D. conanti* in a single encounter exhibited the Waltz (mean duration = 1.5 min, range = 1–2 min; frequency = 2 times).

In stark contrast, no heterospecific encounters reached the sperm transfer stage of courtship. Joint Isolation (JI), or the proportional difference between the number of successful homospecific versus heterospecific encounters, is a mating index commonly used to describe the overall breakdown in courtship between populations or species (Arnold et al., 1996). Success can be judged using the proportion of inseminations as well as that for spermatophore deposition. In this study, $JI_{\text{INSEMINATION}} = 1.21$, whereas $JI_{\text{SPERMATOPHORE}} = 1.46$. Males of both species did exhibit pursuit (Stage 1) and at least initial persuasive behaviors (Stage 2; i.e., Head Rubbing; Table 3) in heterospecific crosses. Male *D. aff. auriculatus* initiated Head Rubbing slightly more frequently (mean = 1.5 times, range = 1–5) than did male *D. conanti* (mean = 0.876, range = 1–3) across photographed heterospecific encounters. Interestingly, I observed what appeared to be Head Rubbing behaviors initiated by two different female *D. aff. auriculatus* in two heterospecific encounters. In one of these encounters, the female *D. aff. auriculatus* approached the male *D. conanti* from behind and slid her body beneath his until their heads were aligned, at which point she pressed the top of her head upwards into his chin and throat. The male then pressed his rostrum downwards and briefly rubbed his head against the top and sides of the female’s head. I did not observe female *D. aff. auriculatus* in homospecific trials, or any female *D. conanti* (SL), initiate Head Rubbing.

I observed aggressive behaviors in one homospecific encounter between *D. aff. auriculatus*, as well as in two heterospecific encounters between male *D. conanti* and a female *D. aff. auriculatus*. The same female *D. aff. auriculatus* was involved in each of these three unique pairings and was the aggressor in the heterospecific encounters, but not in the homospecific encounter. In the homospecific encounter, the male *D. aff. auriculatus* Gaped (Table 1) in front of the female’s head. In each of the heterospecific encounters the female *D. aff. auriculatus* Gaped and then subsequently Bit the male *D. conanti* on the rostrum after he oriented towards and approached her head. I did not observe aggression between *D. conanti* (SL) in homospecific encounters, nor aggression initiated by male or female *D. conanti* (SL) in heterospecific encounters. However, if aggressive behaviors lasted for less than 30 seconds, or if they occurred during the interval not photographed by the GoPro cameras, they may have been missed.

Table 3. Proportion of couples exhibiting each behavior. A total of 12 homospecific encounters were photographed (seven between *D. aff. auriculatus* and five between *D. conanti* [SL]). Fourteen heterospecific encounters were also photographed (eight between a male *D. conanti* [SL] and female *D. aff. auriculatus*, and six between a male *D. aff. auriculatus* and female *D. conanti* [SL]).

Behavior	Homospecific		Heterospecific	
	<i>D. aff. auriculatus</i>	<i>D. conanti</i> (SL)	<i>D. aff. auriculatus</i> male and <i>D. conanti</i> (SL) female	<i>D. conanti</i> (SL) male and <i>D. aff. auriculatus</i> female
Head Rub	0.71	0.60	0.67	0.63
Body Contact	0.86	0.80	1.00	0.88
C-Position	0.57	0.80	0.00	0.00
Waltz	0.57	0.20	0.00	0.00
Tail-Straddle Walk	0.57	0.80	0.00	0.00
Gape	0.14	0.00	0.00	0.25
Bite	0.00	0.00	0.00	0.25

Table 4. Genotypes of known mothers, potential fathers, and offspring from each clutch. The number of offspring successfully genotyped for each locus is included in parentheses. A = locus failed to amplify consistently for this individual across two or more independent amplification attempts. Female *D. aff. auriculatus* #160 mated with male #170 prior to ovipositing Clutch #1, and both male #169 and #170 prior to Clutch #2. Female *D. conanti* (SL) #328 only mated with male #329 in trials.

<i>D. aff. auriculatus</i>						
Locus	Female #160	Male #169	Male #170	Clutch #1	Clutch #2	
<i>Dcon05</i>	353, 293	333, 293	309, 301	(29) 293, 337, 341, 353	(8) 293, 333, 353	
<i>Dcon14</i>	334, 330	342, 294	338, 286	(29) 330, 334	(9) 294, 330, 334, 342	
<i>Dcon16</i>	475, 463	463, 463	459, 439	(29) 463, 467, 475	(9) 463, 475	
<i>Dcon26</i>	296, 296	312, 292	308, 300	(21) 296, 300, 304	(7) 292, 296, 312	
<i>Dcon34</i>	343, 339	A	371, 371	(29) 339, 343	(5) 343	
<i>Dcon36</i>	217, 217	233, 217	217, 217	(29) 217	(9) 217, 233	

<i>D. conanti</i> (SL)			
	Female #328	Male #329	Clutch
<i>Dcon12</i>	276, 276	272, 268	(4) 268, 272, 276
<i>Dcon14</i>	354, 302	322, 298	(4) 298, 302, 322, 354
<i>Dcon26</i>	292, 292	316, 304	(4) 292, 304, 316
<i>Dcon34</i>	403, 375	383, 375	(3) 375, 383, 403
<i>Dcon36</i>	273, 257	269, 253	(4) 253, 257, 269, 273
<i>Dcon40</i>	257, 257	261, 245	(3) 245, 257

Oviposition and insemination.—Females of each species oviposited a single clutch in both 2014 and 2015 ($n = 4$ clutches total). The same female *D. aff. auriculatus* (JYL #160) produced one clutch in each year. The first clutch contained 31 eggs and was oviposited on 7 October, while the second contained ≥ 27 eggs and was oviposited on 15 September. All 31 individuals from the first clutch hatched within 56–59 days, but tissue samples were only collected from 29 individuals. A total of nine hatchlings were produced from the second clutch after 46 days. I collected female *D. aff. auriculatus* #160 from the field on 2 June 2014. She was inseminated by a single male *D. aff. auriculatus* (JYL #170) during courtship trials in 2014, and by two male *D. aff. auriculatus* in 2015 (JYL #169 and #170).

A single female *D. conanti* (SL) oviposited a clutch of at least 14 eggs on 28 October, but by approximately two weeks later had consumed her entire clutch. At least ten of her eggs had been fertilized (i.e., nerulation visible in photos, pre-Stage 16 [Marks and Collazo, 1998]). In 2015 a different female *D. conanti* (SL) (JYL #328) produced a clutch of at least 16 eggs on 22 July. By 7 August a few eggs had turned brown and shrunk in size, but many others were clearly fertilized and developing (i.e., fore and hind limb buds visible, Stage 18 or 19 [Marks and Collazo, 1998]). Only six eggs remained by 15 August, the others presumably consumed by the female. These remaining eggs included two in which no development was visible and four containing embryos that were at approximately Stage 22–24 (i.e., at least some pigmentation visible in the eye [Marks and Collazo, 1998]). To prevent any further loss, I preserved all six eggs in ethanol on 15 August. I used the entirety of each egg to extract genomic DNA for microsatellite genotyping. I collected female *D. conanti* (SL) #328 from the field on 14 February 2015. She was successfully inseminated by a single male *D. conanti* (SL) (JYL# 329) on 19 April 2015.

Excluding potential fathers.—All loci successfully amplified for known mothers, as well as across all but one potential father (i.e., *Dcon34* for *D. aff. auriculatus* #169; Table 4). None of the offspring mismatched the genotype of their mothers. I

observed between one and four alleles in each clutch produced by the female *D. aff. auriculatus*; however, some offspring did not amplify at locus *Dcon26* in the first clutch and at loci *Dcon05*, *Dcon26*, and *Dcon34* in the second clutch (Table 4). Mean observed heterozygosity (\pm standard error) across loci for offspring in the first clutch was 0.580 ± 0.160 , and that for the second was 0.625 ± 0.171 . Despite having successfully inseminated female *D. aff. auriculatus* #160 in both 2014 and 2015, male *D. aff. auriculatus* #170 mismatched all 29 offspring in each clutch at loci *Dcon16*, as well as all genotyped offspring across multiple other loci (Table 4). The alleles of male *D. aff. auriculatus* #169 are represented in the genotypes of each of the nine offspring in the 2015 clutch with no mismatches (Table 4).

Of the six eggs collected from the single clutch from female *D. conanti* (SL) #328, two failed to amplify across four or more loci, and therefore I excluded them from this analysis. The remaining four offspring were genotyped for at least five of the six microsatellite loci, and I observed between two and four alleles across loci in this clutch (Table 4). Mean observed heterozygosity was 0.944 ± 0.056 . The genotypes for all four offspring were concordant with alleles found in male #329 with no unaccounted for alleles.

DISCUSSION

Comparing JI values for this study ($J_{\text{INSEMINATION}} = 1.21$; $J_{\text{SPERMATOPHORE}} = 1.46$) with those of other, similar studies highlights the extreme degree of sexual isolation that I observed between *D. aff. auriculatus* and *D. conanti* (SL) (Fig. 2). It is feasible that these JI values will differ if a larger number of individuals from each population and species are crossed in future studies. However, given that none of the heterospecific couples reached advanced stages of courtship (i.e., Stage 3, sperm transfer; Verrell and Arnold, 1989), it is not likely that isolation estimates will be dramatically lower.

The method of time-lapse photography implemented in this study was able to document multiple interesting behaviors, including both female-initiated courtship and aggression. Female-initiated behaviors have been observed in

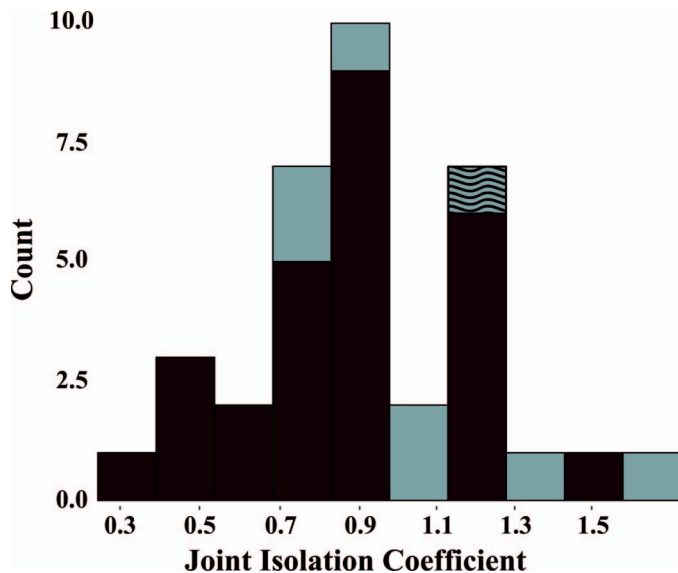


Fig. 2. Joint isolation coefficients from published studies of crosses between species of plethodontids. Joint isolation (JI) coefficients for insemination from crosses between allopatric (black) and sympatric (gray) taxa are represented as a histogram. Results from this study (JI = 1.21) between sympatric *D. aff. auriculatus* and *D. conanti* (SL) are indicated with a patterned fill. Previously published studies represented in this figure include Verrell (1989b, 1990a, 1990b), Verrell and Tilley (1992), Uzendoski and Verrell (1993), Arnold et al. (1996), Herring and Verrell (1996), Kozak (2003), and Mabry and Verrell (2004).

other studies with *Desmognathus* (e.g., nudging [Mabry and Verrell, 2004]), and female small-bodied *Plethodon* perform a number of behaviors which increase the likelihood of pheromone transfer and reception, including Head Rubbing (Dyal, 2006). I observed two different female *D. aff. auriculatus* initiating Head Rubbing in two heterospecific encounters, but larger sample sizes and continuous filming or observation are needed to verify the occurrence and frequency of these behaviors. Aggression has also been documented among species of plethodontids, but, with respect to courtship, any “overt fighting,” including threats, biting, and chasing, appears to occur more often between competing males (Verrell, 1989a). I noted Gaping and Biting (Table 1) between males and females in three different encounters (i.e., one homospecific and two heterospecific), and in each case the aggressor was either a male or a female *D. aff. auriculatus*. In the homospecific encounter, the male *D. aff. auriculatus* performed Gape in front of the female after having initiated multiple instances of Body Contact. This encounter did not progress beyond initial pheromone transfer behaviors. Territorial defense is an unlikely explanation for these behaviors because neither individual was a permanent resident of the enclosure. It would be useful to stage trials between *D. aff. auriculatus* and other species of *Desmognathus* to determine whether the former is generally more aggressive.

I suspect that the Waltz is an important precursor to the Tail-Straddle Walk (Table 1) in *D. aff. auriculatus*, and this may also be the case for other *Desmognathus*. I observed the Waltz in each photographed encounter for *D. aff. auriculatus* that resulted in insemination ($n = 4$). Couples often performed more than one complete rotation, and I observed them to perform the Waltz throughout the enclosure (i.e., this behavior was not necessarily triggered by proximity to a confining wall). Verrell (1997) first described the Waltz in a

courtship study involving populations collected from Aiken and Barnwell counties in South Carolina, which he identified as *D. auriculatus*. Beamer and Lamb’s (2008) survey of lineages of *Desmognathus* across the Coastal Plain suggests that Verrell’s populations are not synonymous with *D. auriculatus* from the topotype locality and therefore should be referred to as *D. cf. auriculatus*. Sequence data (Lamb, 2016; Means et al., in press) demonstrate that populations of *D. cf. auriculatus* from South Carolina are also not synonymous with *D. aff. auriculatus* in Mississippi and Louisiana. It therefore appears that the Waltz occurs in at least two divergent lineages of *Desmognathus* in the Coastal Plain, and that this behavior should be given equal consideration as a discrete courtship behavior in comparative courtship studies. Interestingly, a very similar behavior, the Circular Tail-Straddling-Walk (c-TSW), has been described in *Aneides* (Sapp and Kiemnec-Tyburczy, 2011), a closely related genus of salamanders. Like the Waltz, c-TSW precedes the linear Tail-Straddle Walk that is typical of plethodontids, but, unlike the Waltz, in c-TSW the male’s chin is in contact with the base of the female’s tail (see fig. 1 in Sapp and Kiemnec-Tyburczy, 2011).

Microsatellites (Adams et al., 2005) and allozymes (Tilley and Hausman, 1976; Houck et al., 1985) have been used to determine paternity among species of *Desmognathus* within the *ochrophaeus* complex, but this study is the first to do so for *Desmognathus* in the Gulf Coastal Plain. The genotypes belonging to the clutch produced by female *D. aff. auriculatus* #160 in 2014 suggest that an unknown male must have fertilized this female in the field prior to her capture in June 2014, making these offspring the result of sperm which was stored for at least 127 days (4 mo 5 days). The 2015 clutch produced by the female *D. aff. auriculatus* appears to have been sired by the captive male #169 (Table 4), and therefore she must have stored sperm for at least 149 days (4 mo 27 days). Although it is possible that female #160 stored viable sperm (i.e., capable of fertilizing eggs) between seasons from an unknown male with whom she mated prior to capture (Houck and Schwenk, 1984; Eddy et al., 2015), this outcome seems improbable given the close match between paternal alleles and the ones found in male #169. Similarly, the four offspring in the clutch produced by female *D. conanti* (SL) #328 also appear to be sired by a single captive male, *D. conanti* (SL) #329 (Table 4), and this female must have stored sperm for at least 94 days (3 mo 3 days). There were four or fewer alleles per locus per clutch (Table 4) and the probability of genotyping error was low (i.e., allele sizes were confirmed across at least two amplification attempts). Therefore, assuming that there were no mutations or null alleles (Tennesen and Zamudio, 2003), it is unlikely that any of the three clutches were sired by more than one male.

The behaviors described herein, along with the results of the paternity exclusion analyses, further our understanding of the reproductive ecologies and behaviors of *Desmognathus* in the Gulf Coastal Plain. *Desmognathus aff. auriculatus* and *D. conanti* (SL) from the Pearl and Pascagoula rivers exhibit a high degree of sexual isolation and only appear to exchange initial pheromone transfer behaviors in laboratory courtship trials. Offspring that were the result of homospecific matings indicate that female *D. aff. auriculatus* and *D. conanti* (SL) are capable of storing viable sperm for similar minimum durations, 4 mo and 5 days and 3 mo 3 days, respectively. These minimum estimates are similar to the three-month sperm storage duration suggested by Marynick (1971) for populations of *Desmognathus* from Louisiana. Although no

hybridization occurred between *D. aff. auriculatus* and *D. conanti* (SL), the microsatellite loci used here could be applied in other studies to examine later stages of both pre-zygotic (i.e., sperm competition or exclusion) and post-zygotic isolation (i.e., reduced offspring fitness) among other species and populations of *Desmognathus*.

ACKNOWLEDGMENTS

This work was funded by a National Science Foundation (NSF) Graduate Research Fellowship under Grant No. 0940712 and an NSF “Molecules to Muscles” GK-12 Fellowship Award No. 0947944 through the University of Southern Mississippi. I thank D. Beamer, G. Brown, C. Huntzinger, R. Jones, J. Lee, K. Narum, W. Selman, and A. Sinclair for their help in the field or in identifying potential collection localities. I am grateful to the J. Schaefer Lab for use of their GoPro™ HERO3 cameras. I also thank B. Morris, B. Kreiser, and C. Qualls for their editorial assistance, S. Arnold for our discussions pertaining to this manuscript, and T. Herman for his advice with respect to salamander husbandry. Scientific collection permits were provided by the Mississippi Department of Wildlife, Fisheries and Parks (Permits 0422131 and 0422141). All work was conducted in accordance with the appropriate institutional animal care guidelines (Permit 11061301).

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