

# Hybridization of Two Darter Species Native to Central Virginia

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**Abstract:** Spawning behavior and hybridization of *Etheostoma nigrum* and *E. olmstedii* were studied in the lab using wild individuals collected from populations in central Virginia. Hybridization is common in the genus *Etheostoma*, but there have been conflicting results in studies involving *E. nigrum* and *E. olmstedii*. The question is pertinent to central Virginia because populations with characteristics intermediate of the two species occur there, and it may be that these are hybrids. We collected both darter species in the wild and placed various inter- and intra-specific mating pairs in small aquaria. Males spent more time than females in nuptial behavior (24.1% v. 7.5%), the percentages were not different between species, but *E. nigrum* males spent more time courting *E. olmstedii* females than they did their own species (32% v. 12.4%). Viable eggs were produced in 6 of 24 tanks, including two inter-specific crosses. In both inter-specific crosses the female was *E. olmstedii* and the male was *E. nigrum*. Fry were produced from all six successful matings, but 100% mortality occurred by week four, probably because we did not provide a suitable diet.

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**Key words:** *Etheostoma*, hybridization

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Johnny darters (*Etheostoma nigrum*) and tessellated darters (*Etheostoma olmstedii*) inhabit streams and rivers along the Atlantic slope from southern Canada to North Carolina, with *E. olmstedii* extending into southern Georgia (Jenkins and Burkhead 1993). Simons (1992) determined that they are sister species. They can be distinguished morphologically based on the presence of an incomplete infraorbital (IO) canal in *E. nigrum* (complete in *E. olmstedii*), and a mode of 11 dorsal rays in *E. nigrum* (13–14 in *E. olmstedii*; Jenkins and Burkhead 1993).

Spawning behavior is similar in the two species. Males create a nest under stones or wood, and females, if successfully courted by the males, enter the nest, invert, and deposit adhesive eggs on the underside of the nest material. Males fertilize the eggs, chase away the female, and then guard the nest from predators (Hankinson 1908, 1919, 1932; Winn 1958a, 1958b; Speare 1965; Jenkins and Burkhead 1993).

Hybridization seems common in the genus *Etheostoma* (Hubbs 1955), but there have been conflicting results in studies involving *E. nigrum* x *E. olmstedii* crosses. For example, McAllister et al. (1972) used electrophoresis and phenotypic characteristics to conclude that hybridization was indeed occurring in populations in Ontario, Canada. Stone (1947) studied these same populations and based on character states, concluded that there was no hybridization. Research has also been inconsistent regarding the possible extent of introgression between species (Hubbs 1955).

The status of the two species in Virginia is uncertain. Jenkins and Burkhead (1993) described “problematic” darter populations

in the Falling River of the James River drainage. These populations have characters intermediate to those of *E. nigrum* and *E. olmstedii*. “Good” populations of *E. nigrum* are found predominantly west of this region and “good” populations of *E. olmstedii* to the east (Jenkins and Burkhead 1993).

Clark (1978) studied the problematic populations in Virginia and concluded that they represented introgressive hybrids because they had morphological character-frequency distributions intermediate to those of *E. nigrum* and *E. olmstedii*. Clark (1978) concluded that *E. nigrum* colonized the Falling River by means of stream piracy from Piedmont tributaries of the James River and that the subsequent co-occurrence of this species with *E. olmstedii* led to hybridization. Environmental disruption, as Clark (1978) observed, has been described as a typical factor that may lead to a hybrid swarm. Hubbs (1955) stated that hybrids can be made between practically any two darter species under appropriate conditions. However, Hubbs (1955) also observed that most hybrids have been sterile and thus introgression was rare.

Falls (1982) disagreed with Clark (1978) regarding hybridization in the Virginia populations of *E. nigrum* and *E. olmstedii*. Falls (1982) used starch gel electrophoresis and reported that there was no evidence of hybridization. The most prominent electrophoretic difference observed between the two species was the presence of the *pgi-la* allele. Falls (1982) found no intermediates, suggesting that neither hybridization nor introgression was occurring. Although darter progeny from a natural, inter-specific cross can exhibit characteristics intermediate to those of the parent species

(Hubbs 1959), Falls (1982) concluded that the morphological intermediates found by Clark (1978) were an example of character convergence of *E. olmstedii* to *E. nigrum*, not hybridization.

The objective of this research was to determine if *E. nigrum* and *E. olmstedii* obtained from two Virginia streams will hybridize. We are unaware of any research that has attempted to cross these two species under experimental conditions. We did this by attempting inter- and intra-specific crosses in the lab using wild-caught specimens. We also conducted behavioral observations to evaluate and quantify inter- and intra-specific nuptial behavior.

## Methods

### Species Collection

Individuals of each species (40 *E. olmstedii* and 29 *E. nigrum*) were collected from streams in central Virginia using backpack electrofishing. Collection locations for the two species were based on maps in Jenkins and Burkhead (1993). All *E. nigrum* specimens were collected from Rockets Wild Boar Creek at Rt. 673 in Goochland County (16 March 2005) and all *E. olmstedii* from Falling Creek at Rt. 1 in Hanover County (12 and 21 March 2005). We also attempted to collect fish from the “problematic” populations reported by Jenkins and Burkhead (1993) in order to examine the potential for introgression, but we could locate very few darters of any species in the sampled drainages.

### Species Confirmation

Identification of specimens collected from the wild was based on geographical locale and phenotypic characteristics. The most useful characters for distinguishing the species include (1) IO canals almost always interrupted in *E. nigrum* and uninterrupted in *E. olmstedii*, (2) number of IO pores (7 in *E. nigrum* and 8 in *E. olmstedii*), and (3) second dorsal rays modally 11 in *E. nigrum* and 13–14 in *E. olmstedii* (Jenkins and Burkhead 1993). We based our identifications on only the IO canals and dorsal ray counts because counting IO pores required that specimens be sacrificed. By placing anesthetized (MS-222) fish in water, we were able to observe the IO canal and dorsal rays, confirming the identification of all individuals used in the study. Gender for both species was determined by examining genital papillae (females have short, bi-lobed genital papillae, compared to single lobed in males).

### Fish Culture

Darters were held for one to two weeks prior to the experiment (depending on when they were collected) in two 80-liter holding tanks, one for each species. Six replicates of each possible species combination (*E. nigrum* x *E. nigrum*, *E. olmstedii* x *E. olmstedii*, *E. nigrum* female x *E. olmstedii* male, and *E. nigrum* male x *E. olm-*

*stedii* female) were made by placing a male and female into one of 24, 9.5-liter aquaria. Water temperature was held between 16 C and 17.9 C throughout the duration of the study. Each tank was filled with about 1 cm of gravel and a tile that was positioned in a fashion that would allow eggs to be laid on its underside. Fish were fed frozen bloodworms twice daily. Photoperiod was set at 13 hours light, 11 hours dark, which is approximately the natural photoperiod during the April-May breeding season in central Virginia (Jenkins and Burkhead 1993).

### Behavioral Observations

Male and female behavior in each tank was observed during daylight hours. At the beginning of each viewing session a scan observation (5–10 s) was done for each tank. Tanks in which fish were visible were then viewed for 10.5-minute focal observations. If multiple tanks were showing activity, tanks that had been recorded the fewest times in the past were chosen. This procedure resulted in all tanks being viewed approximately equally over the course of the study (mean = 83.1 min/tank, SD = 7.5).

During observations, the male and female within each tank were observed continuously and the amount of time spent in each of a variety of behaviors was recorded. Data from focal and scan observations were tabulated for each tank, providing an estimate of the activity budget for each fish in each tank (% of time spent in each behavior). Average time across all scans in one tank was treated as a replicate. To simplify the analysis, the detailed behavioral data for each tank was collapsed into the percentage of time spent in any type of nuptial behavior. We were interested in determining if activity budgets (% of time spent in nuptial behavior) varied by gender, species, and type of cross (intra- or inter-specific). We used a 2 x 2 x 2 factorial ( $N = 6$ ) to test for main and interaction effects, after applying an Arc-sin square-root transportation to normalize the percent nuptial behavior data from each tank.

### Spawning

Tanks were examined twice daily during April with a flashlight to check for recently-laid eggs under each spawning tile. If eggs were found the adults were immediately removed from the tank to prevent egg cannibalism. Eggs were counted, photographed, and checked daily until they hatched. Eggs infected with fungus were removed. Once eggs hatched, fry were fed brine shrimp eggs and paramecia as suggested by Linder (1958).

## Results

### Behavioral Descriptions

A total of 1,995 minutes of focal observations and 190 individual scans were completed during the study. Based on focal obser-

vations, there was strong evidence that males spent more time engaged in nuptial behavior than females (males = 24.1%, SE = 3.7%, females = 7.5%, SE = 2.3%; by ANOVA, main effect for gender,  $F = 15.71$ ,  $df_1 = 1$ ,  $df_2 = 40$ ;  $P = 0.0003$ ). This was primarily due to the males spending more time in the nest, attempting to lure females.

The two species spent about the same amount of time engaged in nuptial behavior (by ANOVA; main effect of species,  $F = 0.248$ ,  $df_1 = 1$ ,  $df_2 = 40$ ;  $P = 0.62$ ). *E. nigrum* engaged in nuptial behavior an average of 17.2% (SE = 3.8%) of the time during the observation periods, whereas *E. olmstedii* averaged 14.3% (SE = 3.1%).

There was no effect of type of cross on frequency of nuptial behavior ( $F = 0.165$ ,  $df_1 = 1$ ,  $df_2 = 40$ ,  $P = 0.69$ ). When paired with a mate of the same species, fish spent an average of 13.9% (SE = 2.5%) of their time in nuptial behavior, and about 17.8% (SE = 4.2%) of the time when paired with a mate of the other species.

There were no significant two-way interactions in the ANOVA ( $P > 0.60$  for all 2-way interactions), indicating that *E. nigrum* males behaved similarly (% nuptial behavior = 25.5%, SE = 5.98%) to *E. olmstedii* males (% nuptial behavior = 22.7%, SE = 4.53%), and that females also behaved similarly to each other (*E. nigrum* females = 8.99%, SE = 3.47%, *E. olmstedii* females = 6.0%, SE = 2.69).

The three-way interaction was not significant ( $F = 2.618$ ,  $df_1 = 1$ ,  $df_2 = 40$ ,  $p = 0.11$ ). However, certain three-way comparisons were of particular interest and were examined in more detail. For example, we noticed during observations that *E. nigrum* males spent more time in nuptial behavior (32%, SE = 10.5%) when paired with *E. olmstedii* females than they did when paired with females of their own species (12.4% of the time, SE = 5.2%). The difference was statistically significant at  $\alpha = 0.1$  ( $F = 2.85$ ,  $df_1 = 1$ ,  $df_2 = 40$ ,  $P = 0.097$ ). Scan observations also support the conclusion that *E. nigrum* males preferred *E. olmstedii* females to their own species. *E. nigrum* males paired with *E. olmstedii* females were observed in nuptial behavior during 55% of the scans, but only 16% when paired with their own species.

### Spawning and Development

Eggs were produced in 6 of 24 tanks: 2 involving inter-specific crosses, 3 involving *E. olmstedii* x *E. olmstedii*, and 1 involving *E. nigrum* x *E. nigrum*. In both inter-specific crosses the female was *E. olmstedii* and the male was *E. nigrum*.

The average number of eggs per clutch was 99 (SE = 18.8) across the 6 tanks (Table 1), but many of these were lost to fungus prior to hatching. Eggs were approximately 2.5 mm in diameter and were transparent with a yellowish tint. Hatchlings were approximately 5 mm in length and practically transparent with faint markings along their bodies, similar to markings on adults. It was

**Table 1.** Number of successful (eggs produced) crosses involving two darter species native to central Virginia and number of eggs produced by each successful cross. There were six attempted crosses for each species combination. "Unknown" indicates that all eggs were cannibalized before they could be enumerated.

Species combination	N Successful	Eggs laid
<i>E. olmstedii</i> male x <i>E. olmstedii</i> female	3	147,38,78
<i>E. nigrum</i> male x <i>E. nigrum</i> female	1	Unknown
<i>E. nigrum</i> male x <i>E. olmstedii</i> female	2	120,112
<i>E. nigrum</i> female x <i>E. olmstedii</i> male	0	NA <sup>a</sup>

a. Not Applicable

difficult to determine how many eggs successfully hatched because hatchlings were so small that they hid among the gravel, but it was at least 2–5 per tank. Although we fed hatchlings brine shrimp eggs and paramecia, 100% mortality occurred by week four in all tanks. We assume that fry did not obtain sufficient food and died as their yolk sac was absorbed.

### Discussion

Both intra- and inter-specific crosses produced hatchlings, indicating that the species can hybridize. Both successful inter-specific crosses occurred with *E. nigrum* males and *E. olmstedii* females, perhaps indicating that hybridization occurs only in this combination.

Focal and scan observations help to explain why these results may have occurred. During focal observations, *E. nigrum* males spent more time in nuptial behavior while interacting with *E. olmstedii* females than they did while interacting with their own species (33% vs. 18% of the time in nuptial behavior, respectively). Scan observations showed that *E. nigrum* males and *E. olmstedii* females spent significantly more time in nuptial behavior than did individuals in any other cross.

Hubbs (1955) stated that any hybrid combination could be made between any two darter species under appropriate laboratory conditions, but it is still unclear whether a hybrid between *E. nigrum* and *E. olmstedii* occurs in nature. The specimens used in this study were taken from the wild just two weeks prior to laboratory spawning. This suggests that when living sympatrically (in the wild or in the laboratory), hybridization will occur between the two species. According to Simons (1992), *E. olmstedii* and *E. nigrum* are sister species, suggesting that hybridization is probable. Based on these lines of evidence, we believe that hybridization probably does occur in nature.

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