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**Title: Preference for conspecifics evolves earlier in males than females in a sexually dimorphic radiation of fishes**

Running title:

Male mate choice in early behavioral isolation

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TCM designed the study, assisted in data collection and analysis, and led the writing of the manuscript. JMG, MDM, and PJC assisted in data collection, analysis, and writing of the manuscript.

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# **Preference for conspecifics evolves earlier in males than females in a sexually dimorphic radiation of fishes**

## **Abstract**

Speciation by sexual selection is generally modeled as the co-evolution of female preferences and elaborate male ornaments leading to behavioral (sexual) reproductive isolation. One prediction of these models is that female preference for conspecific males should evolve earlier than male preference for conspecific females in sexually dimorphic species with male ornaments. We tested that prediction in darters, a diverse group of freshwater fishes with sexually dimorphic ornamentation. Focusing on the earliest stages of divergence, we tested preference for conspecific mates in males and females of seven closely related species pairs. Contrary to expectation, male preference for conspecific females was significantly greater than female preference for conspecific males. Males in four of the fourteen species significantly preferred conspecific females; whereas, females in no species significantly preferred conspecific males. Relationships between the strength of preference for conspecifics and genetic distance revealed no difference in slope between males and females but a significant difference in intercept, also suggesting that male preference evolves earlier than females'. Our results are consistent with other recent studies in darters and suggest that the co-evolution of female preferences and male ornaments may not best explain the earliest stages of behavioral isolation in this lineage.

**Keywords:** Etheostoma, female choice, male choice, reproductive isolation, sexual selection, speciation

## **Introduction**

Identifying the barriers to reproduction that define species boundaries, determining when those barriers arise over the course of divergence, and inferring the evolutionary mechanisms responsible constitute major goals of speciation research. One important reproductive barrier in animals is behavioral (sexual) isolation (Coyne & Orr 2004). Behavioral isolation is a reduction in gene flow due to differences in mating signals and mate preferences, which can diverge among populations in at least three ways. One way is through a process of local adaptation, whereby different mating signals and/or preferences are favored in distinct environments (e.g., ecological speciation, sensory drive; Schluter 2001, Boughman 2002). Another is drift in signals or preferences combined with a genetic correlation between the two; any change in one due to chance will cause a change in the other due to genetic correlation (Lande 1981). A third mechanism is mutation, if a novel attractive variant arises and fixes in one geographically isolated population and not another; here too, a genetic correlation can result in the co-evolution of mating signals and preferences leading to behavioral isolation (Mendelson et al. 2014). Current consensus favors local adaptation as the most likely path by which behavioral isolation arises (Ritchie 2007, Safran et al. 2013, Servedio and Bürger 2014, Mendelson et al. 2014, Servedio and Boughman 2017).

Many models describing the evolution of behavioral isolation identify sexual selection as the primary force that acts in concert with ecological selection, drift, and mutation to cause divergence, and the emphasis in these sexual selection models is almost exclusively on the co-evolution of female preferences and exaggerated male secondary sexual traits (ornaments). The hypothesis that behavioral isolation in sexually dimorphic lineages is driven primarily by female preference for conspecific male ornaments is based on the traditional assumption that males are less choosy than females. Females are thought to invest more heavily in reproduction, and this asymmetry in the cost of reproduction is traditionally linked to greater choosiness in females (Darwin 1871, Bateman 1948, Andersson 1994). Moreover, models of speciation by sexual selection are often attempting to explain large radiations of species with

diverse male ornaments (e.g., Kraajeveld et al. 2011), lineages that would seem to fit the paradigm of traditional sex roles. Yet, much of what we know about the phenotypic and genetic architecture of behavioral isolation comes from *Drosophila* and *Lepidoptera*, in which *male* preference for conspecific female traits plays an important role in maintaining species boundaries (Cobb and Jallon 1990, Coyne et al. 1994, Coyne and Oyama 1995, Linn and Roelofs 1995, Jiggins et al. 2001). Thus an important goal is to reconcile a theoretical focus on female preference with empirical data on the importance of male preference in behavioral isolation.

Edward and Chapman (2011) emphasize that most sexual selection theory fails to account for the energy required by males to attract and compete for mates, and rather focuses almost exclusively on gamete investment, parental care, and variance in female quality. Males in many species with exaggerated male ornaments invest heavily in mate guarding and male-male competition, in addition to metabolic investment in the ornaments themselves. These energy expenditures can reduce males' capacity to mate, which in turn could favor male choosiness (Edward and Chapman 2011). Male mate choice now has been demonstrated in many polygynous species with exaggerated male ornaments or displays (e.g. Hill 1993, Simmons et al. 1994, Saether et al. 2001, Werner and Lotem 2003, Godin and Auld 2013). Thus, just as female preferences within species are thought to extend to preferences between species and drive behavioral isolation (e.g., Ryan and Rand 1993), so could male preferences within species extend to interspecific interactions and play an important role in behavioral isolation, even in species with exaggerated sexual dimorphism.

Here we test the prediction that female choice plays the dominant role in the evolution of behavioral isolation in sexually dimorphic lineages with exaggerated male ornaments. We focused on a group of highly sexually dimorphic fishes ("darters") in which males exhibit elaborate nuptial coloration and for which behavioral isolation appears to evolve to completion earlier than other reproductive barriers (Mendelson 2003, Mendelson et al. 2007, Williams and

Mendelson 2014, Martin and Mendelson 2016a). Traditional sexual selection theory predicts that female darters will be choosier than males; however, male darters invest considerable energy in reproduction. In addition to displaying potentially costly nuptial colors (Olson and Owens 1998, Ciccotto et al. 2014a, Kemp et al. 2012), males engage in intense ritualized contests for females, with frequent fin displays, circling, and chasing. In several darter species, males migrate in the spring prior to females, from deeper winter habitats into shallower spawning habitats (Simon and Wallus 2006), setting the stage for intense territorial competition prior to mating. Additionally, in most of the focal species, adult sex ratios in the field are typically skewed towards greater numbers of females (pers. obs.), which may indicate an operational sex ratio favoring greater male choosiness (Emlen and Oring 1977). Finally, a number of recent studies demonstrate a preference for conspecific females in male darters (Ciccotto et al. 2013, Zhou et al. 2015, Martin and Mendelson 2016a, Moran et al. 2017, Roberts and Mendelson 2017).

We examined seven pairs of closely related, allopatric species that vary in genetic distance, i.e., in the amount of time two lineages have been diverging independently (Figure 1). The species pairs represent very early stages of divergence, before behavioral isolation is complete (Martin and Mendelson 2016a, b). Unlike previous studies that examined more distantly related (Mendelson 2003, Mendelson et al. 2007) or sympatric darter species (Williams and Mendelson 2010, 2011, Roberts and Mendelson 2017, Roberts et al. 2017), we focused on species pairs at earlier stages of divergence. Our objective was to observe behavioral isolation at its onset, and without the confounding effect of reinforcement. Simulating secondary contact among recently diverged allopatric lineages is not a crystal ball into the future (e.g., unpredictable factors can affect species interactions in sympatry), but rather a window into the past, revealing how behavior has evolved during early stages of speciation and whether and how the sexes differ in the evolution of preferences that could impact gene flow.

For each species pair, we used standard dichotomous mate choice trials to measure the strength of female preference for conspecific males and the strength of male preference for conspecific females. We tested predictions of the hypothesis that speciation is driven by the co-evolution of female preferences and male signals. Specifically, we tested whether female preference for conspecific males appears at smaller genetic distances than male preference for conspecific females, and whether female preference is consistently larger than male preference across all genetic distances. We found, contrary to expectation, that male preference for conspecific females was on average larger at these early stages of divergence and more likely to be significant than female preference for conspecific males.

## **Methods**

### *Study system*

Darters are a clade of freshwater fishes consisting of four (Smith et al. 2014) or five (Near et al. 2011) genera and approximately 200 species. Darters are endemic to North America, occurring primarily in small to medium mid-elevation streams. Males of most darter species are distinguished from females during the breeding season by elaborate nuptial coloration, though males of some lineages are less colorful but exhibit fleshy knobs (egg mimics) at the tips of the fin rays during the breeding season (Page 1983, Kuehne and Barbour 1983). We focused on a subclade of darters characterized by colorful males and drab females (Figure 1). Darters are externally fertilizing pair-spawners. In the focal species, a gravid female searches for suitable egg deposition sites on rocks or in vegetation while a male closely follows and guards the female by chasing away encroaching males. When a female locates a suitable site, the pair quivers rapidly for 1-2 seconds to release gametes; encroaching males may rush in for a group spawn (pers. obs., see Fuller 2003). Females of the focal species release one egg per quivering bout. Coated with an adherent that keeps it affixed to the substrate, the egg is left to develop in situ, and neither males nor females engage in parental care (Page et al. 1985).

### *Species pair selection*

Focal species were selected to represent phylogenetically independent pairs of allopatric species that span a range of genetic distance (Figure 1, Supplementary Table 1). Phylogenetic independence helps ensure that no two species pairs share overlapping evolutionary history. Allopatric species were chosen to remove potential effects of reinforcement (i.e., a strengthening of conspecific preferences due to selection against hybrids in sympatry, e.g., Butlin 1987); preferences observed in our study therefore should represent the by-product of independent evolutionary trajectories. However, all focal species are sympatric with one or more other darter species, so the effect of indirect or “cascade” reinforcement (Ortiz-Barrientos et al. 2009) cannot be ruled out. Focal species pairs can produce viable hybrid offspring in the lab, with male hybrid sterility appearing in more distantly related focal pairs (Martin and Mendelson 2016b, 2017).

### *Fish collection and maintenance*

Individuals from one population per species were collected from the wild during the breeding season, March - May, over the course of six years spanning 2011-2016. Fishes were captured by kick-seining and transported live in aerated coolers to the University of Maryland Baltimore County. Upon arrival, fish were treated in their coolers with Binox (Jungle Laboratories) for 24 hours to reduce the introduction of foreign pathogens. They were then measured and housed individually on a recirculating aquarium system (Aquatic Habitats, Inc., Apopka, FL, USA) with temperature set to 14 degrees C (+/- 2 degrees) and pH appropriate to the habitat from which the species was collected (range 7.5-8.5). Fish were fed live blackworms and frozen bloodworms daily.

### *Preference trials*



Males and females of all species were tested for conspecific preference in a standard dichotomous choice assay (O'Rourke and Mendelson 2010, Williams and Mendelson 2013, Dougherty and Shuker 2014). Focal individuals were placed in a 37.8L tank ("focal tank") flanked by two 9.5L "stimulus" tanks containing a conspecific or a heterospecific individual of the opposite sex; the heterospecific individual was of the other species in the pair (Figure 1). The focal tank was marked externally with a 10-cm and 5-cm "preference zone" on both ends of the long side of the tank immediately adjacent to each stimulus tank. The three-tank unit was wrapped on three sides with black material, and focal and stimulus tanks were separated by opaque partitions until the onset of the trial. Tanks were illuminated by two full-spectrum fluorescent bulbs (Colormax™ T-5 Aqualight, Coralife®, Franklin, WI, U.S.A.) and two 100W incandescent light bulbs on either end of the unit. Overhead room lights were turned off, and each tank unit plus one observer were obstructed with black curtains to minimize visual disturbance. Each tank contained a layer of mixed aquarium gravel and an airstone.

After focal and stimulus individuals were placed in their respective tanks, opaque partitions were removed, and an observer sat quietly and recorded behavior using jWatcher™ (Blumstein et al. 2000). After the focal fish entered each 10-cm preference zone at least once and returned to the center, "neutral" zone, it was considered acclimated and the observer recorded the amount of time the focal fish spent in the 5-cm preference zones for 20 minutes. Fish were then returned to their home tanks. Using time spent in the 5-cm zone as an estimate of preference, strength of preference for conspecific mates (SOP) was estimated as

$$SOP = T_C / (T_C + T_H),$$

where  $T_C$  is time spent in the 5-cm conspecific preference zone and  $T_H$  is time spent in the 5-cm heterospecific preference zone. SOP therefore ranges from zero to one, with zero indicating complete preference for heterospecifics and one indicating complete preference for

conspecifics. Individuals that spent no time in either of the 5-cm preference zones were excluded from analysis.

The two individuals in a stimulus pair were size-matched to the extent possible while ensuring that no stimulus pair was used more than once. Focal individuals also were used as stimulus individuals, and vice versa, but individuals were never exposed to a particular individual more than once, either as a focal or stimulus. If used as a focal, an individual was given a minimum of 24 hours before being used as a stimulus, and vice versa.

Only visual signals could be communicated in this experimental design. Several studies have shown that darters exhibit preferences based on visual cues alone (Fuller 2003, Williams and Mendelson 2010, 2011, 2013, Williams et al. 2013, Ciccotto et al. 2013, Ciccotto and Mendelson 2016, Roberts and Mendelson 2017, Roberts et al. 2017); moreover, darters are stream-dwelling fish for which olfactory cues may be relatively ineffective. Importantly, however, we compare our results with those of a previous study testing mate choice in the same focal species pairs in artificial streams, where individuals had full physical access to mates and expressed their preferences by spawning (Martin and Mendelson 2016b, see below).

#### *Artificial stream assays*

Mating behaviors for each species pair also were quantified in free-spawning assays in a previous study (Martin and Mendelson 2016a); we used data from these assays to compare with the results of the dichotomous trials. Briefly, free-spawning assays took place in temperature-controlled artificial stream tanks (1.25 x 0.6 x 0.6 m) that mimic stream flow. Each trial consisted of 20 fish in total, five males and five females of each species, with three trials for each species pair. Females of different species were marked with elastomer dye (VIE; Northwest Marine Technology, Inc., Shaw Island, WA, U.S.A.), either anterior or posterior to the spinous dorsal fin, to aid in identification during observations. Males of different species were easily distinguished by eye and left unmarked with the exception of *E. cervus*-*E. pyrrhogaster*.

The location of the dye was alternated between species across trials. After at least 8 hours of acclimation time, fish were observed between the hours of 0800 and 1700 for at least 4 consecutive hours. Each replicate trial was observed for a total of 8 hours over the course of two days.

Courting and spawning behavior was recorded in real time by MDM and used to generate indices of female spawning preference (i.e., preference for conspecific males) and male courting preference (i.e., preference for conspecific females). Data from these trials were re-analyzed here to produce indices that scale from one to zero. Similar to Martin and Mendelson (2016a), female spawning preference for each species (FC) was quantified using spawning data while controlling for the number of courting attempts by conspecific or heterospecific males.

$$FC_i = (S_C/C_C) / ((S_C/C_C) + (S_{HM}/C_{HM})),$$

where  $C_C$  is the number of courting attempts by males of species  $i$  toward females of species  $i$  and  $C_{HM}$  is the number of courting attempts by heterospecific males toward females of species  $i$ ;  $S_C$  is the number of conspecific spawning events and  $S_{HM}$  is the number of heterospecific spawning events by females of species  $i$ . Because the relevant parameters are proportions and can be skewed heavily by values of zero for  $S_C$ , raw data from the three trials per species pair were averaged to yield parameter estimates. Male courting preference was calculated similarly, as

$$MC_i = C_C / (C_C + C_{HF}),$$

where  $C_C$  is the number of conspecific courting attempts by males of species  $i$  and  $C_{HF}$  is the number of courting attempts by males of species  $i$  toward heterospecific females. Male courting

was defined as a male approaching a female, whether or not she allowed him to follow closely or fled (Martin and Mendelson 2016a).

### *Genetic distance*

Strength of preference was compared with three different estimates of genetic distance.

Cytochrome b sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) and analyzed in PAUP\* to generate maximum-likelihood based genetic distances (data from Smith et al. 2016). Because mitochondrial DNA can be prone to introgression generally (Shaw 2002, Funk and Omland 2003) and specifically in darters (Bossu and Near 2009), amplified fragment length polymorphism (AFLP) data generated by Mendelson and Wong (2010) and analyzed by Smith et al. (2016) were used for genome-wide distance estimates. In addition, nuclear sequences were obtained from GenBank, from a 1,314 bp intron of the recombination activating protein 1 (RAG1). RAG1 sequences were aligned and analyzed in MEGA6 (Peterson et al. 2013). Genetic distance was estimated as the number of base substitutions per site using the Kimura 2-parameter model; rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

### *Statistical analysis*

We compared the amount of time spent with conspecifics versus heterospecifics for both sexes in all species. All data sets were tested for normality using a Kolmogorov-Smirnov test. Either a paired t-test or a Wilcoxon signed rank test was used, depending on whether the data met the assumptions of normality.

To compare the strength of preference for conspecifics between females and males, we calculated SOP for each individual and averaged SOP for females ( $SOP_F$ ) and males ( $SOP_M$ ) within each species (Table 1). SOP measures for each species are not independent, however, as each is part of a species pair. Therefore, we averaged  $SOP_F$  and  $SOP_M$  for each species pair

(Table 1) and used a paired t-test to compare them. Indices from the stream data (FC and MC) also were averaged across the two species in each pair (Table 1), and a paired t-test was used to compare them.

We used the average  $SOP_F$  and  $SOP_M$  for each species pair to test for a correlation between genetic distance and  $SOP_F$  and  $SOP_M$ . Relationships were estimated using linear models (lm) in R (R Core Team 2017) with  $SOP_F$  (or  $SOP_M$ ) as the dependent variable and genetic distance as a covariate. Significance of the relationship between genetic distance and  $SOP_F$  (or  $SOP_M$ ) was tested separately for each of the three measures of genetic distance (cytb, AFLP, RAG1).

To test for a difference in the slopes and intercepts of the relationships between  $SOP_F$  and genetic distance and  $SOP_M$  and genetic distance, we used an analysis of covariance with average SOP as the dependent variable, sex as the factor, and genetic distance as the covariate. Our first model tested for an effect of genetic distance, sex, and their interaction on SOP. We then ran a second model testing only the effect of genetic distance and sex (minus the interaction). We ran an analysis of variance to test for a difference between the two models. A significant interaction between sex and genetic distance in model 1 would indicate a difference in the slopes; a nonsignificant interaction between sex and genetic distance in model 1 coupled with a significant effect of sex in model 2, and no difference between the two models, would indicate a significant difference in the intercepts between  $SOP_F$  and  $SOP_M$ .

All protocols and procedures were ethically reviewed and approved by the UMBC Institutional Animal Care and Use Committee as protocol number TM010841518.

## Results

Females in no species spent significantly more time with conspecific than heterospecific males (Table 1, Supplementary Table 2). Female *Etheostoma flavum* spent more time with conspecific males over those of *E. etnieri* ( $t = 1.973$ ,  $df = 14$ ,  $p = 0.069$ ); however, the difference was not

significant at an alpha-level of  $p=0.05$ . Interestingly, this species pair also had the greatest difference in male color pattern, as measured with the male color difference index of Martin and Mendelson (2014), and it exhibited the greatest genetic distance (as measured by cytochrome b and RAG1) in our analysis. Female *E. flavum* also significantly preferred conspecific males in a previous study when given a choice between conspecifics and a more closely related parapatric congener, *E. duryi* (Martin and Mendelson 2013).

Males in four species spent significantly more time with conspecific females than heterospecific (Table 1). Males of *E. ramseyi* ( $t = 2.177$ ,  $df = 16$ ,  $p = 0.045$ ), *E. rupestre* ( $t = 2.948$ ,  $df = 10$ ,  $p = 0.015$ ), *E. coosae* ( $V = 108$ ,  $p = 0.004$ ), and *E. flavum* ( $V = 93$ ,  $p = 0.009$ ) spent significantly more time with conspecific females than females of their heterospecific pair. Three of these species belong to the most genetically distant pairs in our study, which also exhibited the highest estimates of behavioral isolation in the stream assays of Martin and Mendelson (2016a). No species pair was symmetric in male preference, that is, each species with a significant male preference for conspecifics belongs to a unique species pair (Table 1); thus, male preference in only one species of a pair appears to be sufficient to generate an observable degree of overall behavioral isolation (Martin and Mendelson 2016a). Males in one species, *E. lynceum*, spent significantly more time with heterospecific females ( $V = 14$ ,  $p = 0.007$ ).

On average, the strength of preference for conspecific mates (SOP) was  $0.51 \pm 0.02$  (mean  $\pm$  SE) for females and  $0.57 \pm 0.04$  for males in the dichotomous choice trials. SOP was significantly greater for males than for females ( $t = -3.360$ ,  $df = 6$ ,  $p = 0.015$ , Figure 2a).

Using data from the previously reported artificial stream trials, the index of female spawning preference for conspecifics, corrected for the number of courting attempts by males, was  $FC = 0.57 \pm 0.03$ . Male courting preference for conspecific females (MC) was  $0.63 \pm 0.04$ . Male courting preference was significantly greater than female spawning preference in stream trials ( $t = 3.857$ ,  $df = 6$ ,  $p = 0.008$ , Figure 2b). No significant difference was detected

between  $SOP_F$  in dichotomous choice trials and female spawning preference (FC) in stream trials ( $t = 1.238$ ,  $df = 6$ ,  $p = 0.262$ ), and no significant difference was detected between  $SOP_M$  in dichotomous choice trials and male courting preference (MC) in stream trials ( $t = 1.546$ ,  $df = 6$ ,  $p = 0.173$ ), suggesting dichotomous choice trials are reasonable approximations of behavior in unrestricted conditions.

No significant correlation was detected between any measure of genetic distance and average SOP for either females (cytb adjusted  $R^2 = 0.075$ ,  $F = 1.48$ ,  $p = 0.278$ , Figure 3; AFLP adjusted  $R^2 = -0.141$ ,  $F = 0.257$ ,  $p = 0.634$ ; RAG1 adjusted  $R^2 = -0.165$ ,  $F = 0.148$ ,  $p = 0.716$ ) or males (cytb adjusted  $R^2 = -0.195$ ,  $F = 0.020$ ,  $p = 0.894$ , Figure 3; AFLP adjusted  $R^2 = -0.138$ ,  $F = 0.273$ ,  $p = 0.623$ ; RAG1 adjusted  $R^2 = -0.131$ ,  $F = 0.306$ ,  $p = 0.604$ ). No significant difference was detected between males and females in the slopes of the relationships between genetic distance and average SOP; however, the intercept of the relationship between genetic distance and average SOP was significantly different between males and females for cytochrome b (males: 0.57, females: 0.46,  $F = 5.03$ ,  $p = 0.047$ ) and nearly so for AFLP (males: 0.60, females: 0.54,  $F = 4.80$ ,  $p = 0.051$ ) and RAG1 (males: 0.60, females: 0.49,  $F = 4.57$ ,  $p = 0.056$ ), with the intercept for males consistently greater than that for females.

## Discussion

Quantifying mate preferences in males and females of fourteen closely related, allopatric darter species, we tested the hypothesis that female preference drives the evolution of behavioral isolation in the early stages of speciation. We found that females in only one species had a weak preference for conspecific males; whereas, males in four species significantly preferred conspecific females. Males in a fifth species significantly preferred heterospecific females. On average, male preference for conspecific females was significantly greater than female preference for conspecific males. The slope of the relationship between strength of preference and genetic distance, which approximates the rate of evolution of SOP (e.g., Coyne and Orr

1989, Mendelson 2003), was not different between males and females, but the relationships did differ in intercept, suggesting male preference is stronger at the earliest stages of divergence. Results of the dichotomous choice trials were corroborated by data from a previous study of artificial stream trials, in which individuals had full access to mates, where male courting preference for conspecific females was greater than female spawning preferences for conspecific males. Our results therefore do not support the hypothesis that female preference drives the evolution of behavioral isolation and rather suggests that male preference for conspecific mates evolves earlier than females'.

One explanation for our results is that dichotomous mate choice trials are better suited to revealing male choice than female choice in these species. Indeed, females in the focal species generally search for oviposition sites while males search for females; therefore, males may be more likely to approach females (as in a dichotomous choice test) than vice versa. However, males did not spend significantly more total time associating with females in the 5-cm preference zones as compared to females did with males in any species (paired t-test comparing ( $T_C + T_H$ ) of males and females;  $t = -0.910$ ,  $df = 6$ ,  $p = 0.398$ ), and in two sympatric darter species with the same spawning mode, females in dichotomous choice trials strongly prefer conspecific males (Williams and Mendelson 2010) as well as motorized models painted to resemble conspecifics (Williams and Mendelson 2011). Moreover, our results are consistent with other recent findings in darters. Ciccotto et al. (2013) and Roberts and Mendelson (2017) reported significant male preference for conspecific females in dichotomous choice tests, although these species pairs were more distantly related than the focal species. Zhou et al. (2015) concluded that male choice, rather than female choice, is responsible for behavioral isolation between the closely related darter species *E. spectabile* and *E. caeruleum*. Moran et al. (2017) also concluded that male preference for conspecifics is stronger than females' among recently elevated species in the *E. spectabile* group. Finally, our finding that male preference for conspecific females is greater than female preference in artificial stream assays, where



individuals have full physical access, further supports the conclusion that male preference for conspecific mates is stronger at early stages of divergence than females’.

If our results are not an artefact of experimental design, multiple hypotheses could resolve the disparity between theoretical treatments of speciation by sexual selection, which suggest a leading role for female preferences, and our results. First, males in sexually dimorphic species may be choosier than traditional theory suggests (Amundsen 2000, Bonduriansky 2001, Clutton-Brock 2007, Edward and Chapman 2011). Male darters display potentially costly nuptial colors and compete intensely for females (see above), an energy investment that could favor male mate choice (Edward and Chapman 2011). Another resolution with traditional sexual selection theory is that female darters may be choosy, but not with respect to the phenotypes that distinguish species in this study. Phenotypes that distinguish males of two species represent just one axis of variation along which females might express preferences. Although some studies of darters failed to detect female preference for color within species (Pyron 1995, Fuller 2003, Ciccotto et al. 2014b) or for conspecifics (Martin and Mendelson 2013, Zhou et al. 2015), Williams et al. (2013) found that female *E. barrenense* prefer one intraspecific male color variant over another, and female *E. barrenense* and *E. zonale* both prefer conspecific males (Williams and Mendelson 2010) and conspecific coloration (Williams and Mendelson 2011). Female *E. nigripinne* overwhelmingly prefer conspecific males over males of the allopatric congener *E. smithi* (O’Rourke and Mendelson 2010), and female *E. flavum* significantly prefer conspecific males over males of the allopatric congener *E. duryi* (Martin and Mendelson 2013). Thus, female darters exhibit mate choice in certain contexts, so hypothesizing a role for female preference in darter speciation is reasonable. Females in the focal species may have strong preferences, but perhaps for phenotypes that vary more within than between species; such preferences would not be expressed in this experimental design and would fail to reduce gene flow upon secondary contact.

The species-specific phenotypes that explain male preference for conspecific females are unknown. Unlike males, which differ clearly and quantitatively among species in nuptial coloration (Gumm and Mendelson 2011, Martin and Mendelson 2016b), females generally lack chromatic coloration and are difficult to distinguish at the species level. Female darters do exhibit melanistic patterns that could be species-specific, and species can differ in subtle aspects of body shape (Figure 1); however, preliminary analyses reveal no correlation between species differences in female melanin pattern or body shape and male strength of preference (Martin and Mendelson, unpubl. data). Females might exhibit species-specific behavioral signals or cues, but this has not been tested.

Another remaining question is why females in these species pairs do not prefer conspecific males, even though male color patterns are so distinctive. One reason could be that male ornaments are diverging according to a mutation-order process, whereby different but similarly attractive ornaments arise and fix in allopatric populations (Mendelson et al. 2014). If these novel variants are universally attractive to females in multiple populations, only geographic isolation prevents their spread, and females will not prefer local males. Female preferences can diverge among populations in a mutation-order scenario due to a Fisherian process of indirect selection, but only under certain conditions, and only after a lag period (Mendelson et al. 2014). Female preferences for conspecific ornaments thus would lag behind divergence in the ornaments themselves, leading to the pattern we observed of divergence in male signals without a concomitant divergence in female preference; however, this still would not explain the comparatively rapid evolution of male preference for female conspecifics.

A non-mutually exclusive hypothesis is that male preferences for conspecific female phenotypes, as well as the female phenotypes themselves, are pleiotropic byproducts of a concerted evolution of male competitive responses and male signals that arises due to male-male competition. In many species, male signals are used in both contests and courtship (Berglund et al. 1996), and thus in principle could respond as much to the receiver psychology

of males as to that of females. The nuptial colors of male darters, for example, appear to be used in contests with rival males more often and more intensely than in courting females (pers. obs.), and several studies demonstrate that male darters have strong preferences for (i.e., bias their aggression toward) conspecific color patterns (Williams and Mendelson 2013, Martin and Mendelson 2016a, Moran et al. 2017). If a novel ornament is more intimidating than the average signal, males with that ornament will win access to females, and selection will favor its spread. Males that preferentially respond to the novel ornament also increase their reproductive success by identifying successful males and either challenging their access to females or engaging in group spawns. Darwin (1871) speculated that the muted expression of male ornaments in females of sexually dimorphic species is due to what we now call pleiotropy. Strong male aggressive responses evolving in concert with conspecific male ornaments therefore could manifest as preference for the pleiotropic, muted versions of those ornaments expressed in females. Muted pleiotropy also could explain the difference between males and females in strength of preference, if female preference for conspecific male ornaments is a muted version of male preference.

The role of male-male competition in driving speciation by sexual selection is gaining attention (e.g. Seehausen and Schluter 2004, Dijkstra et al. 2007, Qvarnstrom et al. 2012, McCullough et al. 2016, Tinghitella et al. in press), but a plausible link between competitive responses within the sexes and mate preferences between the sexes has posed a challenge to its full acceptance as a mechanism of speciation by sexual selection. One overlooked link is simply that the high energy investment of male-male competition can favor male mate choice (Edward and Chapman 2011), leading to behavioral isolation via male preference for conspecific females. Another is the hypothesis we suggest here, that a heightened response in males to conspecific male ornaments can lead to male preference for conspecific females, if male ornament alleles have pleiotropic effects on female phenotypes.

## **Conclusion**

We tested the hypothesis that behavioral isolation in darters is driven primarily by female preference for conspecific male ornaments. Contrary to our expectation, but consistent with a number of recent studies, we found that male preference for conspecific females is stronger than female preference for conspecific males at the earliest stages of divergence. We propose a hypothesis of concerted evolution of male competitive responses (preferences) and male signals with pleiotropic effects on male preferences for female signals as an alternative to hypotheses that focus exclusively on the co-evolution of female preferences and male signals. Although that hypothesis remains to be fully developed and tested, our data nonetheless suggest that the receiver psychology of males, whether in contests, courtship, or both, plays an important role in the evolution of behavioral isolation between species. Male choice is known to contribute to behavioral isolation in *Drosophila* and *Lepidoptera*; our results suggest it may be just as important in highly dimorphic species with exaggerated male secondary sexual traits.

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**Table 1.** Strength of preference for conspecifics for females and males of fourteen species of *Etheostoma*. SOP<sub>F</sub> and SOP<sub>M</sub> indicate mean SOP (+/- SE) for females and males, respectively, in each species. Asterisks indicate a significant difference in the amount of time associating with conspecifics versus heterospecifics. FC<sub>stream</sub> is mean female spawning preference and MC<sub>stream</sub> is mean male courting preference for conspecific mates in artificial stream trials (Martin and Mendelson 2016a). All indices were averaged across the two species in each pair. GD is genetic distance based on cytochrome b sequence data (AFLP and RAG1 data in Supplementary Table 1).

Species	SOP <sub>F</sub>	SOP <sub>M</sub>	FC <sub>stream</sub>	MC <sub>stream</sub>	Average SOP <sub>F</sub>	Average SOP <sub>M</sub>	Average FC <sub>stream</sub>	Average MC <sub>stream</sub>	GD <sub>cytb</sub>
<i>E. cervus</i>	0.37 (0.08)	0.59 (0.08)	0.51	0.60	0.43	0.59	0.54	0.56	0.009
<i>E. pyrrhogaster</i>	0.50 (0.08)	0.58 (0.08)	0.57	0.53					
<i>E. ramseyi</i>	0.48 (0.07)	0.64 (0.07)*	0.45	0.57	0.53	0.59	0.47	0.54	0.100
<i>E. tallapoosae</i>	0.58 (0.07)	0.55 (0.10)	0.50	0.51					
<i>E. raneyi</i>	0.48 (0.06)	0.50 (0.06)	0.41	0.54	0.51	0.54	0.45	0.57	0.163
<i>E. zonistium</i>	0.52 (0.08)	0.57 (0.05)	0.48	0.61					
<i>E. barrenense</i>	0.57 (0.09)	0.60 (0.11)	0.37	0.80	0.55	0.60	0.54	0.63	0.228
<i>E. orientale</i>	0.52 (0.08)	0.60 (0.10)	0.72	0.47					
<i>E. lynceum</i>	0.46 (0.10)	0.21 (0.08)*	0.78	0.61	0.44	0.49	0.69	0.70	0.276
<i>E. rupestre</i>	0.43 (0.12)	0.77 (0.07)*	0.60	0.79					
<i>E. baileyi</i>	0.60 (0.09)	0.48 (0.09)	0.68	0.83	0.49	0.60	0.68	0.71	0.371
<i>E. coosae</i>	0.38 (0.07)	0.71 (0.08)*	0.67	0.60					
<i>E. etnieri</i>	0.53 (0.08)	0.51 (0.08)	0.54	0.61	0.62	0.62	0.61	0.68	0.381
<i>E. flavum</i>	0.69 (0.07)	0.72 (0.07)*	0.69	0.75					

## Figure Legends

Figure 1. Cladogram depicting phylogenetic relationships among focal species based on AFLP fragment data (Mendelson & Wong 2010, Smith et al. 2014) with focal species pairs indicated in bold and images of males in breeding coloration (left) and females of each focal species.

Figure 2. A. Strength of preference for conspecifics (SOP) for females and males of seven species pairs of *Etheostoma* in dichotomous mate choice trials. SOP ranges from zero (complete association preference for heterospecifics) to one (complete association preference for conspecifics), with SOP = 0.5 indicating no preference. B. Female spawning preference (FC) and male courting preference (MC) for conspecific mates in artificial stream trials (data from Martin and Mendelson 2016a). FC and MC also range from zero to one. Asterisks indicate significant difference.

Figure 3. Relationship between genetic distance based on cytochrome b sequence and average SOP for females (SOP<sub>F</sub>, open circles, dashed line) and males (SOP<sub>M</sub>, closed circles, solid line) for seven phylogenetically independent species pairs of *Etheostoma*. No significant correlation was detected between genetic distance and SOP for females or males; however, a significant difference between the intercepts for SOP<sub>F</sub> and SOP<sub>M</sub> was detected.