Male behaviour predicts trait divergence and the evolution of reproductive isolation in darters (Percidae: *Etheostoma*)

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The diversity of secondary sexual signals across animals has led evolutionary biologists to hypothesize an important role for sexual selection in the process of speciation. Testing this hypothesis typically focuses on male–female mating interactions and divergence in female mate preferences between populations, leading to behavioural reproductive isolation. While informative, these tests rarely account for the potential contributions of male–male competitive interactions and male mate preferences to speciation. By incorporating male and female behavioural data across a genus of colourful freshwater fishes (Percidae: *Etheostoma*), our results suggest coevolution between male signals and aggressive responses and a potential role for male mating biases alongside female mating biases in the evolution of behavioural isolation between allopatric species. These surprising results imply an important function for male behaviours in trait divergence and the evolution of reproductive isolation, suggesting that a broader view of sexual selection is vital to addressing its role in speciation.

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young avian hybrid zone, male—male competition probably contributes to habitat segregation and ecological reproductive isolation between *Ficedula* flycatcher species (Vallin, Rice, Bailey, Husby, & Qvarnström, 2012). These studies demonstrate that male—male competition can facilitate speciation and divergence in male agonistic signals as well as in aggressive responses.

Like male—male competition, male mate choice also can lead to divergence and reproductive isolation but is understudied in comparison to female mate choice (Edward & Chapman, 2011). Traditional sex roles describe competitive males vying for the attention of choosy females (Darwin, 1871); however, recent work suggests that sex roles can be dynamic, responding to changing demographic parameters (Forsgren, Amundsen, Borg, & Bjelvenmark, 2004; O’Rourke & Mendelson, 2013) or even reversed, if the costs of mating are larger for males (Berglund & Rosenqvist, 2003). Male mate choice, like female mate choice, can lead to behavioural isolation between species. For example, in cliff and boulder ecotypes of the marine snail *Littorina saxatilis*, cliff males distinguish the mucous trails of females and preferentially follow trails laid down by cliff females (Johannesson et al., 2008). In *Heliconius* butterflies, males strongly prefer the wing markings of conspecific females, thus contributing to behavioural isolation (Jiggins, Naish, & Mallet, 2001). These and similar data have prompted many calls to examine female mate choice, male competition and male mate choice simultaneously in the context of sexual selection (Edward & Chapman, 2011; Hunt, Breuker, Sadowski, & Moore, 2009; Wong & Candolin, 2005). Here, we apply that comprehensive study design to examine how interactions within and between the sexes contribute to speciation by sexual selection in a diverse genus of fish.

*Etheostoma* is a genus of over 150 freshwater fish species commonly called darters, which are native to North American streams and lakes. Darters exhibit conspicuous, species-specific nuptial colour patterns and other elaborate secondary sexual traits, strongly implicating sexual selection in the process of speciation (e.g., Mendelson, 2003; Fig. 1). Darters are seasonal breeders, typically spawning in the early spring. In some species, males are territorial and defend and display from nests; however, in most species, including the focal taxa, males initiate courtship by approaching a female and swimming alongside her, occasionally performing a lateral display and/or quivering for short bursts. A female can either accept a courting male or swim away. If she accepts, the pair will swim in tandem while the female jabs her nose along the substrate, investigating potential ovipositon sites. During the search, other males may approach and attempt to disrupt the pair. These intense competitive interactions can, but do not necessarily, result in the replacement of the initial male. Once a suitable ovipositon site is located, the male and female simultaneously release gametes, which are left unattended. Mating interactions in darters therefore create opportunities for female choice, male—male competition and male choice.

Indeed, previous experimental results support a role for different mechanisms of sexual selection in darters. Male body colour is subject to female choice in at least one species of darter (Williams, Gumm, & Mendelson, 2013), and females prefer conspecific to heterospecific coloration in at least one sympatric species pair (Williams & Mendelson, 2010, 2011). Mate guarding ability is associated with mating success in *Etheostoma caeruleum* (Fuller, 2002), and nests of more aggressive males have more eggs present in *Etheostoma flavibellum* (O’Rourke & Mendelson, 2014), indicating that male competition influences mating success in darters. Furthermore, males in some species bias their aggression towards conspecific males (Zhou, Loew, & Fuller, 2015). Male preference for conspecific over heterospecific females also has been documented in darters (Ciccotto, Gumm, & Mendelson, 2013; Williams & Mendelson, 2010; Zhou et al., 2015), providing evidence of male mate choice. Together, these results suggest that female mate choice, male competition and male mate choice simultaneously contribute to sexual selection and potentially behavioural reproductive isolation in darters.

To examine the role of both male and female biases in trait divergence and behavioural isolation, we simulated secondary contact between eight phylogenetically independent, allopatric species pairs of darters. For each pair, we quantified male colour differences and observed interactions in artificial streams to quantify female preference (bias) for conspecific males, male aggression bias towards conspecific males, male preference (bias) for conspecific females and the strength of behavioural isolation. Positive associations between male colour differences, female mating biases and behavioural isolation would be consistent with female mate preferences facilitating divergence in male signals, leading to reproductive isolation. A positive association between male colour differences and male aggression bias would be consistent with male competition contributing to trait divergence. Finally, a positive association between male mating biases and behavioural isolation would be consistent with male mate preferences leading to reproductive isolation. None of these patterns are mutually exclusive.

**METHODS**

**Specimen Collection and Care**

Specimens were collected during the months of March and April in 2009—2012 (localities in Supplementary Table S1). After collection, specimens were transported in aerated coolers to the University of Maryland Baltimore County (Baltimore, MD, U.S.A.). Individuals were housed in same-sex stock tanks and fed live blackworms twice daily. After behavioural assays, individuals were euthanized using an overdose of tricaine methanesulfonate (MS-222; Acros Organics, Geel, Belgium). Specimen collection was approved by the Alabama Department of Conservation and Natural Resources, the Mississippi Department of Wildlife, Fisheries, and Park, the Tennessee Wildlife Resources Agency, the South Carolina Department of Natural Resources and the Kentucky Department of Fish and Wildlife. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Maryland Baltimore County (protocol TM010240811).

**Species Pair Selection**

We chose focal species pairs from three closely related subgenera commonly called snubnose darters and allies (Supplementary Fig. S1; *Ulocentra, Etheostoma* s.s. (i.e. sensu stricto, referring to the subgenus *Etheostoma* within the genus *Etheostoma* and *Nanostoma*). We chose eight pairs such that species within a pair were allopatric and pairs were phylogenetically independent from one another. We assessed phylogenetic independence using a tree constructed using amplified fragment length polymorphisms (AFLP; Mendelson & Wong, 2010). Evolutionary changes along nonoverlapping trajectories can be considered independent and thus allows the use of traditional statistics (Felsenstein, 1985).

**Behavioural Assays**

We simulated secondary contact between allopatric species in the laboratory using free-spawning behavioural assays (e.g. Martin & Mendelson, 2013; Mendelson, 2003; Williams & Mendelson, 2010). Behavioural assays were carried out in temperature-
controlled artificial stream tanks that simulate stream flow. Illumination included two 60 W incandescent bulbs, two T5 fluorescent bulbs (6700K, Colormax; Coralife®; Franklin, WI, U.S.A.) and natural sunlight. Each trial consisted of five males and five females of each species (i.e. 20 fish total). Females in some species pairs could not be distinguished and were thus tagged using a small amount of visible implant elastomer (VIE; Northwest Marine Technology, Inc., Shaw Island, WA, U.S.A.) either anterior or posterior to the spiny dorsal fin. Males were easily distinguished and remained unmarked in all species pairs except Etheostoma cervus—E. pyrrhogaster. The location of these marks was switched in replicate trials. After being placed in the artificial stream tank, fish were allowed to acclimate for a minimum of 8 h prior to observation. Observations for each replicate trial occurred over 2 days during the hours of 0800 and 1700 for at least 4 sequential hours per day. Three replicate trials were conducted for each species pair.

Aggression, courting and spawning events were recorded and scored as either conspecific or heterospecific. Behavioural scoring was conducted in real time by a single observer (M.D.M.). Observations were made through the transparent wall of the tank without use of a blind, as darter fish appear to habituate quickly to the presence of an observer (e.g. Martin & Mendelson, 2013; Mendelson, 2003; Williams & Mendelson, 2010). All focal species lack swim bladders and spend much of their time on the substrate without excessive motion or interaction. Thus, behaviours were recorded from all 20 individuals in the tank simultaneously without the use of focal sampling or other methods, as these may have limited our ability to score meaningful behaviours. Male—male aggression typically consisted of chasing but also included lateral fin displays and bites on occasion; all observed occurrences of these behaviours were recorded as aggression. Courting in darters consists of males first approaching a female, then swimming in tandem as the female searches for a suitable oviposition site. Courting was recorded whether the female allowed the male to swim in tandem or not. Spawning is an easily recognizable behaviour in darters; in all cases, heterospecific spawning occurred to some degree.

**Behavioural Indices**

We used aggression, courting and spawning data to estimate behavioural indices. Each index ranged from -1 to 1, indicating either a heterospecific behavioural bias (i.e. negative values), no behavioural bias (i.e. 0), or a conspecific behavioural bias (i.e. positive values). Each index is based on the estimation of behavioural isolation proposed by Stalker (1942):

\[
BI = \frac{s_c - s_h}{s_c + s_h}
\]

where \(s_c\) and \(s_h\) are the number of conspecific and heterospecific spawning events, respectively. Male aggression bias is calculated as:

\[
\frac{s_m - s_c}{s_m + s_c}
\]
MA = \frac{a_c - a_h}{a_c + a_h} \quad (2)

where \(a_c\) and \(a_h\) are the number of conspecific and heterospecific aggressive interactions (i.e. chases, fin displays and bites), respectively. Male mate choice is calculated as:

\[ \text{MC} = \frac{c_c - c_h}{c_c + c_h} \quad (3) \]

where \(c_c\) and \(c_h\) are the number of conspecific and heterospecific courting events directed from males to females, respectively. And finally, female mate choice bias is calculated as:

\[ \text{FC} = \frac{s_c - s_h}{s_c + s_h} \quad (4) \]

where \(s_c\) and \(s_h\) are the number of conspecific and heterospecific spawning events, respectively, and \(c_c\) and \(c_h\) are the number of conspecific and heterospecific courting events directed from males to females, respectively. Female choice, or acceptance, is considered as the ratio of spawning events to courting events; in other words, how often a female mates with a courting conspecific or heterospecific male. The female mate choice bias index therefore is calculated to control for differences in male courting effort (i.e. even if male effort is low (\(c_h\)), female acceptance may still be high (\(s_h/c_h\)). We computed each index for each trial and averaged them across trials, resulting in a single number for each species pair.

**Colour Differences**

Differences in male colour have been calculated previously and thus methods used here have already been described in detail (Martin & Mendelson, 2014). Briefly, we used visual-system-dependent methods to collect quantitative colour data using digital photography. After euthanasia, we fixed specimens in formalin for no more than 3 min and photographed them under incandescent light using a PowerShot A650 IS (Canon USA, Inc., Melville, NY, U.S.A.). Images were taken in the RAW file format at the same aperture and exposure settings, ignoring the in-camera white balance. We converted the RAW files to TIFF files, linearized the red, green and blue pixel values and converted them to darter cone photometric data (i.e. \(s_c\) and \(s_h\) from equations (1) and (4)). As these indices were derived using some of the same data, the observed significant relationship could be a result of these shared data. To determine whether the observed relationship was an artefact of these shared data, we generated 10 000 simulated data sets for number of courting and spawning events for eight hypothetical species pairs. We calculated behavioural isolation (BI) and female choice bias (FC) using these numbers and assessed correlations between these randomly generated indices. Among 10 000 simulated data sets, 950 produced a correlation coefficient greater than or equal to our actual data (i.e. \(P = 0.0950\)), indicating that the observed relationship between female choice bias and behavioural isolation may in fact be a result of the overlap in data used to calculate each index.

**DISCUSSION**

Our results suggest a role for male behaviour in sexual signal divergence and behavioural isolation in darters. Male sexual signals...
appear to have coevolved with male agonistic responses in allopatry but not with female mating responses, which are often the focus of speciation by sexual selection models (e.g. Fisher, 1930; Lande, 1981). In addition, male courting biases predicted behavioural isolation while female mating biases were only marginally better than randomly generated data at predicting behavioural isolation. Our results therefore portray male darters as simultaneously competitive and choosy, instead of one or the other, and suggest a more important role for male behaviour in trait divergence and behavioural isolation than is generally conceived. While female mate preferences almost certainly contribute to speciation by sexual selection in darters and many other systems, results presented here and elsewhere (Edward & Chapman, 2011; Qvarnström et al., 2012) support a balanced approach to research that considers male biases as well, in both intra- and intersexual interactions.

Male colour differences between darter species correlated with male aggression bias but not with female mating bias, supporting a model of colour divergence by intrasexual selection. Current discussions of speciation by intrasexual selection (male–male competition) focus largely on sympatric interactions (Qvarnström et al., 2012). For example, the agonistic character displacement hypothesis (Grether, 2011; Grether et al., 2009) suggests that aggressive signals and male competitive responses (e.g. competitor recognition templates sensu Grether, 2011) diverge between sympatric species when aggression towards heterospecific males is costly. Divergence in our species pairs has occurred in allopatry; nevertheless, similar mechanisms may lead to the patterns we observe in allopatry. As a by-product of agonistic sympatric isolation.

![Figure 2](image1.png)  
**Figure 2.** Relationships between male colour differences and (a) female choice bias, (b) behavioural isolation and (c) and male aggression bias in *Etheostoma*.

![Figure 3](image2.png)  
**Figure 3.** Male and female contributions to behavioural isolation in *Etheostoma*. Behavioural isolation is predicted by both (a) female choice bias and (b) male choice bias. However, female choice bias was not significantly better than randomly generated data at predicting behavioural isolation (simulation test: $P = 0.095$).
interactions, refined competitive responses could lead to discrimination against allopatric competitors whose signals fall outside the range of rival male signals. Such a process would be analogous to ‘cascade reinforcement’ (Kozak et al., 2015; Ortiz-Barrientos, Grealy, & Nosil, 2009), whereby reinforcement of mating preferences in sympatric congeners can increase the strength of behavioural isolation between congeneric allopatric populations (Pfennig & Rice, 2014; Pfennig & Ryan, 2006). Darters can form diverse communities of up to 12 species (Etner & Sternes, 1993; Page & Burr, 2011), suggesting the potential for cascading agonistic character displacement; however, the prevalence of colour patterns and interspecific aggression in these communities requires further study.

Alternatively, differences in the habitats of allopatric species could contribute to divergence in male agonistic signals if environments differ in ways that affect signal transmission or perception. This pattern is well established for male signals in the context of female mate choice, although these signals also can be used in agonistic interactions (Endler, 1992). In freshwater sticklebacks, males in red-shifted light have black nuptial markings that increase contrast with the background, as compared to males in green-shifted light, which exhibit the ancestral state of red (Boughman, 2001, 2002). The colour of male dewlaps in Anolis lizards has diverged to maximize brightness contrast in populations occupying distinct signalling environments (Leal & Fleshman, 2004; Persons, Fleshman, Frye, & Stimpfl, 1999), and across numerous species of antbirds, the frequency (pitch) of male song is correlated with the sound transmission properties of distinct habitats (Seddon, 2005).

In darters, the extent of sexual dichromatism (colour differences between the sexes within a species) and the presence/absence of nuptial coloration covaries with habitat use across the genus (Bossu & Near, 2015; Ciccotto & Mendelson, 2015). However, among very closely related darter species, such as the focal species examined here, sexual signal differences are not associated with environmental differences (Martin & Mendelson, 2012), and sympatric darter species often express different colour signals in the same habitats (Etner & Sternes, 1993; Page & Burr, 2011). Thus, the importance of ecological differences in generating the patterns revealed here is open to further investigation.

Our results suggest a role for male mate choice, alongside female mate choice, in the evolution of behavioural reproductive isolation. Although females are typically considered the ‘choosy’ sex, male mate choice is potentially widespread (Edward & Chapman, 2011) and has been documented in freshwater fish (Godin & Auld, 2013; Gregorio, Berdan, Kozak, & Fuller, 2012; McKinnon et al., 2012) including darters (Ciccotto et al., 2013; Zhou et al., 2015; but see O’Rourke & Mendelson, 2010). Male choosiness is predicted when males invest substantial energy in reproduction, usually in the form of paternal care (Kokko & Monaghan, 2001; Trivers, 1972). Focal species in our study do not exhibit paternal care; however, costs are incurred by males while courting and prior to spawning, during which males vigorously ward off rivals using displays, bites and chases (M. D. Martin, personal observation; Fuller, 2003; Williams & Mendelson, 2010). In addition, males must maintain their agonistic signals. Nuptial colours in darters are likely a product of carotenoid and other pigments (Fuller, 2003; Porter, Fiumera, & Avise, 2002) and could impose costs via immunological trade-offs (Cloftfelter, Ardia, & McGraw, 2007) or increased predation risk (Moodie, 1972; Olsson, 1993). In contrast, females do not produce costly ornaments and expend virtually no energy soliciting courtship. Because darters are external fertilizers, females also are unlikely to incur any physical trauma in the act of spawning. The main cost of reproduction for females in the focal subgenera is the production of 500–1000 eggs (Weddle & Burr, 1991), which they lay one at a time and allocate across multiple partners. This promiscuous mating system therefore may impose relatively weak selection on female choice compared to polygynous systems in which females allocate many eggs to one or a few males. The energy invested by males in courtship and by females in egg production therefore may balance out, or weigh slightly towards male investment, explaining the significant relationship we observed between male choice bias and behavioural isolation.

Although males may be exerting some degree of mate choice, the trait or traits on which this choice may be based is unclear. In darters, females of different species are typically more similarly coloured than males of different species (M. D. Martin, personal observation). However, other visual cues such as body shape and size could influence interspecific mating decisions. Males of some darter species have been shown to discriminate conspecific and heterospecific females based on visual cues (Ciccotto et al., 2013; Zhou et al., 2015). In addition to visual signals, some darter species also produce acoustic signals in mating contexts (Johnston & Johnson, 2000), but these appear to be limited to males in the Catonotus group, outside of the focal groups examined here. Conspecific and heterospecific chemical cues are known to elicit alarm reactions (Smith, 1979) in darters, but their role in mating decisions has not been assessed. Notably, chemical mating cues have been implicated in other freshwater fish systems (Mason & Ryan, 1997). While acoustic signalling seems unlikely to play a role in male mating decisions in our focal species pairs, some combination of visual and chemical cues may contribute to male mate discrimination, and the contributions of each sensory modality may differ between species pairs.

Several considerations are important when interpreting these results. First, our results are correlative and thus cannot demonstrate causation; however, these unexpected correlations warrant further investigation into the influence of multiple mechanisms of sexual selection on divergence in darters and other natural systems. Complementary studies in each of our focal species examining intra- and intersexual selection could clarify the relative importance of these mechanisms within species and how these may lead to divergence between species (e.g. Zhou et al., 2015). Second, the lack of a relationship between female preferences and behavioural isolation could result from small sample sizes (i.e. N = 8). Although significant correlations were observed among other variables with similar sample sizes, none of these required additional testing using data simulation. Previous studies have provided concrete evidence that female mate choice acts within darter species (Williams et al., 2013) and has diverged between species (Williams & Mendelson, 2010, 2011); however, several others demonstrate weak or absent female mate choice in darters (Ciccotto, Gumm, & Mendelson, 2014; Fuller, 2003; Martin & Mendelson, 2013; Pyron, 1995; Zhou et al., 2015). Additional comparative research could test whether these discrepancies result from species differences in the relative choosiness of each sex. Third, each species is represented here by individuals from a single population. Our sampling design increased taxonomic breadth at the expense of replication within species, making our observations vulnerable to the idiosyncrasies of particular populations. Of particular importance could be the darter community present at each collection site, as these sympatric species could shape the behavioural responses of each population. Without additional sampling, we cannot be certain that the patterns observed here are indicative of patterns across species; however, in so far as population-level patterns represent the early stages of species-level divergence, our results suggest a role for male behaviours in trait divergence and reproductive isolation. Nevertheless, further replication using multiple populations within species would be particularly insightful as we work to clarify the general role that male behaviours play in speciation. Finally, it is unclear to what degree these behavioural responses in males and
females are genetically based or learned. Mate choice copying has been demonstrated in darters (Moran, von Ende, & King, 2013), but the role that previous experience plays in aggressive responses remains untested. Assessing behavioural responses in laboratory-reared individuals could help clarify the genetic and environmental contributions to the behavioural interactions investigated here.

Our investigation suggests two surprising but complementary patterns in darter divergence. First, male signal differences coevolve with male aggressive responses, suggesting a potential role for male–male competition in trait divergence. Second, male mating biases predict behavioural isolation between species, potentially more so than female biases, counter to predictions of traditional sexual selection theory. Results therefore highlight the potential importance of male biases in both intra- and intersexual interactions in the process of speciation by sexual selection. Alongside a growing body of literature, our results therefore support a more comprehensive view of speciation by sexual selection, expanding beyond the traditional focus on female preference for elaborate male ornaments to include the biases of males towards both male and female signals.

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Supplementary Material
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