Male mate choice contributes to behavioural isolation in sexually dimorphic fish with traditional sex roles

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Behavioural isolation between closely related species with exaggerated male mating traits is traditionally thought to be mediated by female preference for conspecific male ornaments. The role of male mate choice in maintaining boundaries between highly sexually dimorphic species is therefore comparatively neglected. However, mounting theoretical and empirical evidence supports the existence of male mate choice both within, and increasingly, between species with exaggerated male ornaments. We therefore tested the role of male mate choice in the maintenance of species boundaries for two sexually dimorphic species of darters (Percidae: Etheostoma). Using dichotomous choice assays, we measured male preferences of sympatric species *Etheosotma barrenense* and *Etheosotma zonale* for size-matched conspecific and heterospecific females, thus reducing the possibility that males would select for general indicators of fecundity. Our results show that males of both species strongly prefer conspecific females. A comparison with published data showed that the strength of preference for conspecific mates is just as strong for males as it is for females in *E. barrenense*. We also estimated the relative contribution of male mate choice, female mate choice and male–male competition to behavioural isolation. We found that male mate choice contributes as much as female mate choice to total behavioural isolation and thus likely plays an important role in behavioural isolation in these sexually dimorphic species. Our results suggest that the contribution of male choice to behavioural isolation should be studied in a larger variety of animal species to appreciate the relative roles of the sexes in the maintenance of species boundaries.

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In many animals, males benefit more than females from increased mating success, leading to a greater intensity of sexual selection on male mating traits (Andersson, 1994; Bateman, 1948). These differences between the sexes are due in part to differences in gamete investment and in part to a lack of male parental care, which leads to higher potential reproductive rates in males and a skewed operational sex ratio, with an overabundance of available males relative to females (Emlen & Oring, 1977). These sex differences are often used to explain the sexual dimorphism common in many polygynous species, where males are indiscriminate and showy while females are choosy and drab. In contrast, mating traits in monomorphic species are hypothesized to have equal intensities of sexual selection (Trail, 1990), or alternatively to be driven by similar natural selective forces between the sexes, with sexual selection playing a weak role in trait evolution (Björklund, 1984).

While parental investment, potential reproductive rates and the operational sex ratio have typically been used to predict mate choice, Edward and Chapman (2011) suggested that mating effort is also an essential factor in the evolution of mate choice. Mating effort includes investments that increase the number of mates available to an individual at the cost of reducing the ability to invest in future matings (Edward & Chapman, 2011). For example, increased investment in mate guarding or courtship displays may increase individual fertilization success, but it also subsequently reduces the ability to invest in future matings (Møller & Birkhead, 1991; Pilastro & Bisazza, 1999). By increasing the cost of mating investment, mating effort therefore can tip the balance in favour of the benefits of exhibiting mate choice. Because mating effort is typically a male investment, male mate choice may evolve in species that do not fit the traditional predictions for male mate choice (e.g. polygynous species with traditional sex roles).

Female choice itself also may create an opportunity for male mate choice to arise. If female preferences for attractive males creates large variation in male mating success, with a majority of males obtaining few or no matings, then a minority of males have the potential to mate with multiple females. Thus, even if the

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operational sex ratio is male biased, female preferences may create a scarcity of favoured males. If a male's capacity to mate with all available females is limited, and the costs of choice do not outweigh its benefit, then mate choice by high-quality males could be favoured (Edward & Chapman, 2011). For example, in the two-spotted goby, *Gobiusculus flavescens*, high-condition males are more successful at attracting mates, more successful in male–male competition and more likely to express mate preferences than low-condition males (Amundsen & Forsgren, 2003).

Male mate choice has been observed in several species of insects (reviewed by Bonduriansky, 2001), fishes (Côte & Hunte, 1989; Rowland, 1982; Sargent, Gross, & Van Den Berghe, 1986) and mammals (Kuester & Paul, 1996; Schwagmeyer & Parker, 1990) among others. However, the traits most often reported to be preferred by males are indicators of fecundity (e.g. large body size), which can be uniform across females of many species (Bonduriansky, 2001). Thus, evidence for male mate preference does not necessarily imply that male mate choice contributes to behavioural isolation (Hochkirch, Gröning, & Bücker, 2007; Kozak, Reisland, & Boughmann, 2009). In other systems, however, male choice appears to be important in maintaining species boundaries, for example, in Lepidoptera (Roelofs & Comeau, 1969), *Drosophila* spp. (von Schilcher & Dow, 1977) and snails (Johannesson et al., 2008). Notably, however, these species do not have exaggerated sexually dimorphic sexual ornamentation. Male preference for conspecific females has been demonstrated in some species of sexually dimorphic fishes (Gregorio, Berdan, Kozak, & Fuller, 2012; Knight & Turner, 1999; West & Kodric-Brown, 2015), but male choice in species with exaggerated male ornaments is rarely implicated in behavioural isolation. Rather, behavioural isolation between such species is thought to be primarily driven by female preference for conspecific male ornaments (Fisher, 1930; Lande, 1981; West-Eberhard, 1983), leaving the role of male choice in the behavioural isolation of sexually dimorphic species a neglected question.

Darters (Percidae: *Etheostoma*) are a diverse group of North American freshwater fish (Page & Burr, 1991), consisting of over 200 species, many of which are characterized by species-specific nuptial coloration (Kuehne & Barbour, 1983; Page, 1983). Coloration is largely limited to males and is expressed primarily during the breeding season (Page, 1983), suggesting that sexual selection has played a role in speciation. As such, much work has been done on the role of female mate choice and male–male competition in reproductive isolation in darters (Fuller, 2003; Martin & Mendelson, 2013; Mendelson, 2003; Mendelson, Imhoff, & Venditti, 2007; Williams & Mendelson, 2010, 2011, 2013). However, some evidence has begun to suggest a potential role for male mating preferences in behavioural isolation for some darter species as well. For example, in a comparative study across several pairs of darter species, Martin and Mendelson (2016) found that male courting preferences better predicted the strength of behavioural isolation than female spawning preferences, and Ciccotto, Gumm, and Mendelson (2013) showed that males of the darter species *Etheostoma luteovinctum* have strong association preferences for conspecific over heterospecific (*Etheostoma hopkinsi*) females.

Sympatric darter species *Etheostoma barrenense* and *Etheostoma zonale* are members of closely related subgenera (Porter, Fiumera, & Avise, 2002) that represent some of the most closely related darter species to co-occur without hybridizing in nature (Hubbs, 1955, 1967; Keck & Near, 2009). Males of the two species exhibit elaborate and divergent nuptial coloration, with male *E. barrenense* displaying primarily red-orange coloration with black blotches fused along the lateral line, while male *E. zonale* have alternating green and yellow bars along the body. Females of both species are drab in comparison to males, although they have some muted coloration and display the patterning of the conspecific males (Fig. 1). The two

species are largely syntopic where they co-occur (Williams & Mendelson, 2014) and display similar courtship and egg-laying behaviours, with neither sex in either species providing parental care (Page, Smith, Burr, & Mayden, 1985). Previous work with these species using dichotomous choice trials demonstrated strong association preferences in females for live conspecific males (Williams & Mendelson, 2010) and for conspecific male colour patterns on painted models (Williams & Mendelson, 2011), suggesting that female preferences contribute to behavioural isolation. In addition, however, in free-spawning assays, males of these two species exclusively courted conspecific females (Williams & Mendelson, 2010), suggesting that male choice also plays a role in behavioural isolation. However, males were not subject to the same dichotomous choice trials as females in this study, so whether assortative male courtship in free-spawning assays was a consequence of male preferences for female phenotypes is not clear.

Male *E. barrenense* and *E. zonale* also have been shown to exhibit increased aggression towards models painted to resemble conspecifics (Williams & Mendelson, 2013) and were more likely to chase away live conspecific males in seminaturalistic artificial stream environments (Williams & Mendelson, 2010), which suggests that male–male interactions also may contribute to behavioural isolation between these species. Therefore, we also considered the potential role of male–male competition in behavioural isolation. Male–male competition may influence speciation by facilitating divergence in male agonistic signals and aggressive responses through negative frequency-dependent selection or by promoting divergence in male traits, behaviours and/or resource use (Qvarnström, Vallin, & Rudh, 2012). For example, selective male exclusion of heterospecific individuals from preferred breeding sites has been shown to contribute to behavioural isolation between two species of *Ficedula* flycatchers (Vallin & Qvarnström, 2011).

In the present study, we sought to determine whether male preference for conspecific female visual signals contributes to behavioural isolation for sympatric darters *E. barrenense* and *E. zonale*, by measuring male association preference for physically and chemically isolated conspecific and heterospecific females. Additionally, we estimated the relative importance of male mate choice, female mate choice and male–male competition to behavioural isolation for this species pair using previously published data on female mate choice and male aggression bias (Williams & Mendelson, 2010) and our own measure of male mate preferences. Our results show strong association preference for conspecific females in males of both species and suggest that male choice can play a central role in the maintenance of species boundaries in this species pair.

METHODS

Fish Collection and Maintenance

We collected *E. barrenense* and *E. zonale* from the East Fork of the Barren River in Monroe Co., Kentucky, U.S.A. (36°44′51″N,
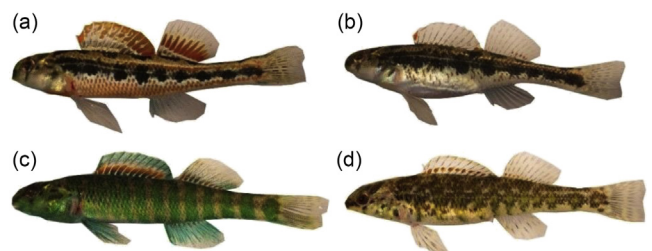

Figure 1. Focal species in breeding colours: (a) male and (b) female *Etheostoma barrenense*; (c) male and (d) female *Etheostoma zonale*.

85°41′49″W) during 9–10 April 2016. We transported fish to the University of Maryland Baltimore County and housed them in a recirculating aquarium system (Aquatic Habitats, Inc., Apopka, FL, U.S.A.). Water temperature, conductivity and pH for fish housing replicated the natural habitat (temperature = 12 °C; conductivity = 550–650 μS; pH = 8.3). We separated fish by sex and species into aquaria lined with gravel and maintained a 12:12 h light:dark cycle. Fish were maintained on a diet of live black worms provided once daily. Male choice trials were completed during 14 April–4 May 2016, coinciding with the natural breeding season (Etnier & Starnes, 1993). All fish tested were sexually receptive based on the presence of nuptial coloration in males and a visibly distended abdomen, indicating gravidity, in females. Permission to collect fish was granted by the Kentucky Department of Fish and Wildlife Resources (permit number SC1611199) and all housing and experimental procedures were approved by the Office for Research Protections and Compliance of the University of Maryland Baltimore County (Animal Welfare Assurance number A3784-01; UMBC IACUC protocol TM01841518).

Preference Trials

Dichotomous choice assays were set up following methods from Williams and Mendelson (2010). A 37.9-litre glass 'test' tank (50 × 25 cm and 30 cm high) was positioned between two 9.6-litre glass 'stimulus' tanks (30 × 15 cm and 20 cm high) so that the long sides of the stimulus tanks were flush against the short sides of the test tank. We marked two 5 cm 'preference zones' at either end of the test tank closest to the stimulus tanks and recorded the time spent in each preference zone during a 15 min trial. Time spent in a preference zone is a good indicator of a female's motivation to mate with the associated stimulus in other fish species (Aspbury & Basolo, 2002; Gonçalves & Oliveira, 2003; Jeswiet & Godin, 2011; Lehtonen & Lindström, 2008) and, in two species of darters, male reproductive success is proportional to the amount of time spent in proximity to a particular female (Zhou, Loew, & Fuller, 2015). Additionally, preferences measured in dichotomous choice assays are in the same direction, although lower in magnitude, as measures of spawning success in artificial streams for several darter species, including *E. barrenense* and *E. zonale* (Ciccotto et al., 2013; Martin & Mendelson, 2013; Mendelson, 2003; Williams & Mendelson, 2010). Therefore, association time appears to be a reasonable measure of preferences in natural encounters.

Both stimulus tanks and the test tank were lined with gravel of equal heights and filled with equivalent depths of water from the aquarium housing, and all tanks were individually aerated. Each stimulus tank was illuminated with an incandescent light source (GE Crystal Clear, A19, 100 W), and a 91 cm full-spectrum light source (Coralife® F/W T-5 Aqualight, 21 W Colormax™ bulb, 21°W 6700 K bulb) spanned all three tanks. Prior to a trial, opaque partitions were placed between each stimulus tank and the test tank, gravel was rinsed and mixed, and water was replaced. While the opaque partitions were in place, we placed one female *E. barrenense* into one stimulus tank and one female *E. zonale* into the other stimulus tank, and introduced a male into the test tank. Once the male began free-swimming activity, the opaque partitions were removed and acclimation began. Acclimation was deemed complete after a male entered both 5 cm preference zones and subsequently entered the 'neutral zone' (i.e. was not in either preference zone). Following acclimation, the 15 min observation began and time spent in each preference zone was recorded using JWatcher™ V1.0 (Blumstein, Evans, & Daniel, 2000).

To control for experimental side bias, each trial alternated which side of the test tank the conspecific female was placed and post hoc analysis indicates that neither species exhibited side bias (two-

tailed Wilcoxon signed-ranks test: *E barrenense*: $Z = -0.76$, $N = 18$, $P = 0.49$; *E. zonale*: $Z = 1.31$, $N = 16$, $P = 0.19$; see Supplementary material 1). We tested preferences for 18 male *E. barrenense* and 16 male *E. zonale* with 33 unique pairs of *E. barrenense* and *E. zonale* females. The one nonunique pairing of female *E. barrenense* and *E. zonale* was shown to a male of each species, so that no female pairing was tested twice for the same species of focal male. Female *E. barrenense* were used in a maximum of two trials while *E. zonale* females were used in one to four trials due to deaths over the course of the trial dates. Post hoc analyses indicated that the repeated use of females had no effect on male preferences for conspecifics (see Supplementary material 1). Stimulus females were size-matched within 15% of their standard length (i.e. snout to caudle peduncle; average ± SD difference between paired females = 1.59 ± 1.05 mm; average standard length of females = 43.97 mm; Supplementary material 2, Table S1). There was no significant size difference between paired stimuli (two-tailed *t* test: *E. barrenense*: $t_{17} = 0.66$, $P = 0.52$; two-tailed Wilcoxon signed-ranks test: *E. zonale*: $Z = 0.72$, $N = 16$, $P = 0.47$; see Supplementary material 1). Male preference for larger and more fecund females has been shown in many taxa (Andersson, 1994; Bondurianszky, 2001; Sargent et al., 1986; Verrell, 1995), thus size matching of females helped ensure male preferences were based on visual differences unrelated to fecundity.

Analysis

Male preference in the dichotomous choice assay was measured and compared to female preference for these species (data from Williams & Mendelson, 2010; see Supplementary material 1). The amount of time spent in either conspecific or heterospecific preference zone was converted into the proportion of time in each zone relative to the entire 15 min trial. Male strength of preference (SOP) for each individual was calculated as

$$SOP = (T_C - T_H)/(T_C + T_H) \tag{1}$$

where T_C is time spent in the conspecific preference zone and T_H is time spent in the heterospecific preference zone. The SOP score can range from +1 (indicating a complete preference for conspecific individuals) to −1 (indicating a complete preference for heterospecific individuals), whereas a score of 0 indicates no preference. Shapiro–Wilk tests and Q–Q plots indicated non-normality for all but one data set; therefore, we used a nonparametric Wilcoxon signed-ranks test to compare male preference for conspecific and heterospecific visual stimuli, and a two-tailed *t* test for the normally distributed data set. We used a Mann–Whitney *U* test to compare male strength of preference to female strength of preference. Data analysis was conducted in R (v.3.2.2; R Core Development Team, 2015).

To estimate the relative role of each sex to total behavioural isolation, we applied methods from Sobel and Chen (2014) to estimate the relative contribution of male mate choice, female mate choice and male–male competition to total behavioural isolation. We first estimated the absolute contribution (AC) of each behavioural barrier, taking into account the order of each contribution to a spawning event so that later acting barriers were adjusted by the weight of earlier acting barriers in behavioural isolation. We followed equation (4S3) in Sobel and Chen (2014) to calculate a combined reproductive isolation (RI$_{total}$) score for multiple sympatric barriers, focusing only on components of behavioural isolation. The absolute contribution of each barrier was then calculated as follows:

$$AC_i = RI_{[1,i]} - RI_{[1,i-1]} \tag{2}$$

where $RI_{[1,i]}$ denotes the combined isolation calculated from the first barrier (1) to the focal barrier (i), and $RI_{[1,i-1]}$ denotes the same calculation omitting the focal barrier (Sobel & Chen, 2014). The relative contribution of each reproductive barrier is estimated as the ratio of its AC to RI_{total} following Ramsey, Bradshaw, and Schemske (2003).

For *E. barrenense* and *E. zonale*, males initiate courtship by approaching a female. Females then have the option to accept or reject a male and, if accepted, other males often attempt to supplant the original male or spawn alongside the primary pair. Thus, we assume the relative order in which male and female behaviour contributes to behavioural isolation in this system to be male mate choice (MC), female mate choice (FC), and then male–male competition (MM). MC is calculated as the average SOP scores for males of both species, FC is the average SOP scores for females of both species, and MM is the average male aggression bias for males of both species (see Supplementary material 1), calculated as

$$MM = (a_c - a_h)/(a_c + a_h) \qquad (3)$$

where a_c and a_h are the mean number of conspecific and heterospecific aggressive interactions, respectively. Data for FC and MM were taken from Williams and Mendelson (2010) (Supplementary material 2, Table S2). Note that focal males were not chemically and physically isolated from stimuli in artificial stream assays as in dichotomous choice assays. While male aggression may be influenced by social interactions in artificial stream assays, Williams and Mendelson (2013) found that male association time with and frequency of fin flares (an aggressive male signal) directed towards painted models did not differ in the presence of a female. Thus, differences in social context between dichotomous choice and artificial stream paradigms should not entirely negate the comparison between these two treatments.

RESULTS

Dichotomous Choice Trials

Both male *E. barrenense* and *E. zonale* spent significantly more time in the conspecific 5 cm preference zone compared to the heterospecific preference zone. Male *E. barrenense* spent a mean $\pm$ SE of $43.4 \pm 6.3\%$ of the total trial time in the conspecific preference zone compared to $11.4 \pm 3.0\%$ of total trial time in the heterospecific preference zone (two-tailed Wilcoxon signed-ranks test: $Z = -3.07$, $N = 18$, $P < 0.001$; Fig. 2a). Male *E. zonale* spent a mean $\pm$ SE of $42.8 \pm 6.8\%$ of the total trial time in the conspecific preference zone and $18.8 \pm 3.8\%$ of the total trial time in the heterospecific preference zone ($Z = -2.12$, $N = 16$, $P < 0.05$; Fig. 2b). In total, 16 out of 18 *E. barrenense* males and 12 out of 16 *E. zonale* males spent more time in the conspecific preference zone than in the heterospecific preference zone. There was no significant difference in the number of males that preferred conspecific females between the two species (two-tailed Fisher's exact test: $P = 0.39$).

Males of both species had SOP scores significantly greater than zero. Mean $\pm$ SE SOP score was 0.54 ± 0.10 for male *E. barrenense* (one-tailed Wilcoxon signed-ranks test: $Z = -3.77$, $N = 18$, $P < 0.001$) and 0.30 ± 0.14 for male *E. zonale* ($Z = -2.14$, $N = 16$, $P < 0.05$). SOP did not significantly differ between males of the two species (two-tailed Mann–Whitney U test: $Z = 1.10$, $N = 34$, $P = 0.28$).

Male and Female Comparison

Male *E. barrenense* tended to spend a larger proportion of total time in the conspecific preference zone as compared to females, but
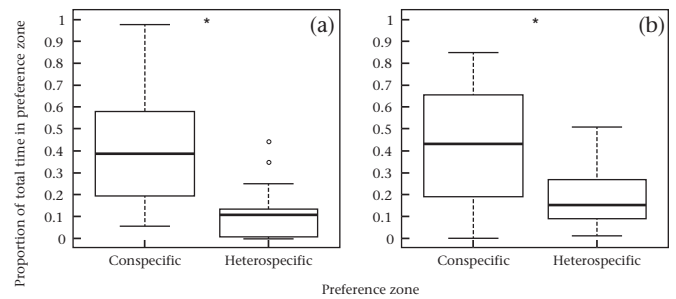
Figure 2. Preference for conspecific females. Box plots indicating the proportion of total time spent by (a) male *E. barrenense* ($N = 18$, $P < 0.001$) and (b) male *E. zonale* ($N = 16$, $P < 0.05$) in the 5 cm conspecific and heterospecific female preference zones. Bars represent medians, boxes indicate upper and lower quartiles, whiskers show sample minima and maxima, and open circles show outliers. *Indicates a significant difference between zones (two-tailed Wilcoxon signed-ranks test).

not significantly so (two-tailed Mann–Whitney U test: $Z = 1.90$, $N = 36$, $P = 0.06$), and both sexes spent a similar proportion of time in the heterospecific preference zone ($Z = 0.98$, $N = 36$, $P = 0.33$; Fig. 3a). Male and female *E. zonale* spent a similar proportion of time in the conspecific preference zones (unpaired two-tailed t test: $t_{33} = -1.03$, $P = 0.31$), but male *E. zonale* spent a significantly larger proportion of total trial time in the heterospecific preference zone than females (two-tailed Mann–Whitney U test: $Z = 2.52$, $N = 34$, $P < 0.05$; Fig. 3b).

Strength of preference did not differ between male ($N = 18$) and female ($N = 18$) *E. barrenense* (two-tailed Mann–Whitney U test: $Z = -0.29$, $P = 0.78$); however, SOP was significantly lower for male *E. zonale* ($N = 16$) compared to female *E. zonale* ($N = 18$) ($Z = 2.31$, $P < 0.05$).

Relative Contribution of Males and Females to Behavioural Isolation

For *E. barrenense* and *E. zonale*, the contribution of each component of behavioural isolation is calculated as an average of the two species isolation indices, resulting in $AC_{MC} = 0.42$, $AC_{FC} = 0.41$, and $AC_{MM} = 0.15$. The combined total reproductive isolation (RI_{total}) due to behavioural barriers was 0.97, making the relative contribution of each behaviour to the total as follows: MC = (0.42/0.97) = 0.43, FC = (0.41/0.97) = 0.42 and MM = (0.15/0.97) = 0.15.

We conducted post hoc effect size and power analysis using the lwr and pwr package in R for each statistical comparison. Power was calculated for the given effect size (d) at an alpha level of 0.05
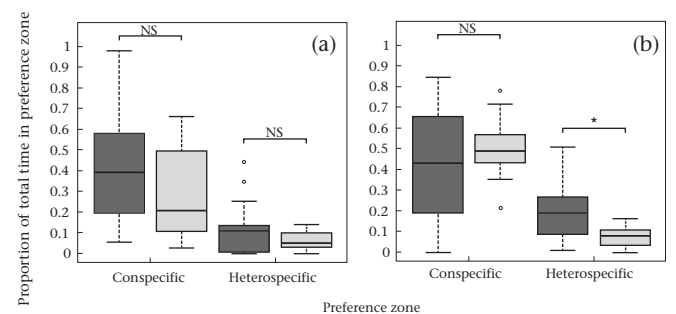
Figure 3. Association times for male and female (a) *E. barrenense* and (b) *E. zonale*. Dark bars represent the proportion of time in a preference zone for males and light bars represent the proportion of time in a preference zone for females. Bars represent medians, boxes indicate upper and lower quartiles, whiskers show sample minima and maxima, and open circles show outliers. *Indicates a significant difference between zones (two-tailed Mann–Whitney U test).

for all statistical comparisons. Additionally, to check whether nonsignificant results were due to lack of power, we determined the sample size needed with an alpha level of 0.05 to achieve power at the recommended 0.80 level (Cohen, 1977; Supplementary material 2, Table S3). Sample sizes ranging from 74 to 7090 would be required to detect a significant result at the recommended power level for the statistical comparisons made. These prohibitively large sample sizes suggest that lack of significance for these comparisons were not due to small sample size (Supplementary material 2, Table S3).

DISCUSSION

While it is becoming increasingly accepted that males exhibit mate choice more often than originally predicted in non-sex-role reversed species (Edward & Chapman, 2011), the role of male choice in behavioural isolation is studied far less often, especially when males are highly ornamented. Here, we found a role for male mating preferences in the maintenance of species boundaries for two sympatric species of darters in which males are elaborate and provide no parental care. Males of both species preferred conspecific over heterospecific females, and in *E. barrenense*, male conspecific preference was not significantly different from that of female preferences. Because females were size-matched in our trials, our results further suggest that male preference is based on female visual signals other than fecundity, although it is possible that unmeasured indicators of female fecundity could affect male preference. Calculating the relative contribution of each sex to total behavioural isolation for this species pair suggests that male mate choice plays at least as large a role as female mate choice in behavioural isolation, and that, overall, male behaviour (male mate choice and male–male competition) accounts for over half of the observed behavioural isolation for this species pair.

Male mate choice may be favoured in darters for several reasons. Mate choice is thought to be favoured when a sex has a relatively low potential reproductive rate (PRR), is underrepresented in the operational sex ratio (OSR) and faces trade-offs between future mating opportunities and increased investment in parental care and/or mating effort (Edward & Chapman, 2011). While PRRs of each sex and the OSR have not been quantified in our focal species, the PRR and OSR reflect investment in parental care (Clutton-Brock & Parker, 1992; Clutton-Brock & Vincent, 1991; Emlen & Oring, 1977), such that the sex that provides more parental care is underrepresented in the OSR (Emlen & Oring, 1977). For example, in the fantail darter, *Etheostoma flabellare*, males provide sole parental care for eggs, and the adult sex ratio (a component of the OSR) in wild populations shows a sharp decrease in male numbers relative to females throughout the breeding season (O'Rourke & Mendelson, 2013). However, neither sex provides parental care in the two focal species in our study, which may create a more equal OSR between the sexes.

Male mating effort also may create opportunities for male mate choice to evolve in darters. In many species of darters, males initiate courtship and only court or guard one female at a time (Fuller, 2003; Martin & Mendelson, 2016; Williams & Mendelson, 2010). Additionally, male darters compete intensely over mates (Fuller, 2003; Williams & Mendelson, 2010) and male guarding ability has been shown to predict spawning success in at least one species of darter (Fuller, 2003). If increasing investment in courtship, competition and/or mate guarding reduces males' future capacity to mate, as seen in other systems (Byrne & Rice, 2006; Møller & Birkhead, 1991; Pilastro & Bisazza, 1999), this increased investment in mating effort by males may favour male mate choice in darters.

How male mate choice within species translates to behavioural isolation between species depends on the female phenotypes that males assess when making a mate choice. In the case of female choice, the phenotypic targets of mate choice are assumed to be elaborate male signals, as has been suggested in the focal species pair, in which females prefer the nuptial coloration of conspecific males (Williams & Mendelson, 2011). It therefore stands to reason that the cues males assess when choosing mates are not the same as the male-specific phenotypes used for female choice. Many studies have found that males prefer female visual signals that indicate fecundity, such as large body size (Bonduriansky, 2001); however, we found that male *E. barrenense* and *E. zonale* preferred conspecific females over size-matched heterospecifics based on visual cues. Female *E. barrenense* and *E. zonale* exhibit species-specific patterning and muted colour, and they differ in some aspects of body shape (Fig. 1). Which of these visual cues, or combination of cues, males use to assess mates in not known, nor whether males might be responding to differences in female behaviour. Future studies are necessary to determine which female phenotypes males are assessing both within and between species.

Divergent male ornaments also have been shown to be important in male–male interactions that contribute to behavioural isolation between species. Male-limited ornaments, such as conspicuous coloration, are often characterized as targets of female choice and separated from male armaments that are used in male–male aggressive interactions (McCullough, Miller, & Emlen, 2016). However, there are several examples of male–male competition mediated by differences in male ornaments that could drive divergence of male traits (Losos, 1985; Qvarnström et al., 2012), and, in some cases, male–male competition has been found to drive colour evolution independent of female choice (Grether, 1996; Zhou & Fuller, 2016). Coevolution of male aggressive responses and coloration has been found in a number of darter species (Martin & Mendelson, 2016) and, for one species pair, behavioural isolation appears to be maintained through competitive exclusion of heterospecific males from receptive females (Zhou et al., 2015). Thus, darters represent an excellent system for future study of the contribution of male–male competition to both ornament divergence and behavioural isolation.

Our results suggest that male behaviour (intra- and interspecific interactions) accounts for over half of total behavioural isolation for the focal species pair. Increased contribution of males to behavioural isolation has been experimentally observed in several monomorphic species of insects despite larger parental investment by females (DeWinter & Rollenhagen, 1990; Lloyd, 1968; von Schilcher & Dow, 1977). One reason male behaviour contributes more than female choice to behavioural isolation is that males in these species initiate courtship. von Schilcher and Dow (1977) hypothesized that in such species, strong female choice initially maintains behavioural isolation between sympatric species, which creates selection on male preferences for homotypic females to avoid wasted mating effort on unreceptive females. Over time, male choice becomes primarily responsible for behavioural isolation simply because males initiate courtship. However, this may not account for all aspects of courtship behaviour in the focal species. Males were never observed to group spawn (i.e. when males spawn alongside the primary male) with heterospecific females in artificial stream assays (Williams & Mendelson, 2010), even though females would be unlikely to reject them, and males in other darter species have been shown to increase fertilization success by spawning alongside dominant males (Fuller, 1999). Further research into the fitness of hybrids from this species pair may reveal hybrid incompatibilities that better explain male preferences, perhaps suggesting that reinforcement (i.e. when hybrid offspring

have reduced fitness) strengthens behavioural isolation for this species pair.

Finally, we found that although male and female SOP were not significantly different for *E. barrenense*, the same was not true for *E. zonale*, in which male SOP was lower than female SOP. One explanation for this result is that female coloration influences male mate choice (Amundsen & Forsgren, 2003; Hill, 1993); although female darters do not express coloration to nearly the same degree as males, some degree of coloration can be observed on females (Kuehne & Barbour, 1983). Red-orange coloration in *E. barrenense* is likely carotenoid based (Gumm & Mendelson, 2011) and may thus indicate aspects of individual condition (Kodric-Brown, 1989). Green coloration on the other hand is produced at least in part by pigment-containing cells called cyanophores (Pearsall, 2005). It is not known to what degree cyanophores are endogenously synthesized or exogenously acquired, and the function of blue/green coloration in darters is unknown. Green coloration may therefore be less informative than red coloration, which could lead to weaker selection for male preferences for green. Differences between sexes in SOP also may be due to different costs of hybridization between species. In crosses between two allopatric darter species, Mendelson et al. (2007) found that the strength of hybrid inviability depends on the direction of the cross (i.e. the species of the female), and stronger conspecific preference in species with higher costs of hybridization has been observed in two species of killifish (Berdan & Fuller, 2012).

Female activity directed towards males also may affect male behaviour and potentially explain the difference observed between species during our trials. Stimuli behaviour was found to affect both the repeatability of preferences and the amount of time spent with stimuli in the guppy *Poecilia reticulata* (Kodric-Brown & Nicoletto, 1997), suggesting that interactions between focal and stimulus individuals could alter male preferences. Female *E. barrenense* may represent a more enticing stimulus to males of both species due to increased reactivity towards both conspecific and heterospecific males (Williams & Mendelson, 2011), explaining the relatively lower SOP for male *E. zonale* than for male *E. barrenense*. Because our study was designed to mirror previously published methods for female *E. barrenense* and *E. zonale,* we did not aim to alter the experimental methods to account for stimulus behaviour. We did, however, note that males did not always associate with the most active stimulus female during trials and overall activity levels appeared lower for *E. barrenense* than for *E. zonale* (N. S. Roberts, personal observation). Nevertheless, as in many dichotomous choice assays, interactions between stimulus and focal individuals are often impossible to control without the use of researcher-controlled stimuli (e.g. animated videos), and our results must be interpreted with this in mind.

In conclusion, we show that males demonstrated strong conspecific mate preferences in two species of sexually dimorphic fish with traditional sex roles and that conspecific preference was as strong for males as for females in one species. Estimates of the contribution of male mate choice, female mate choice and male–male interactions to total behavioural isolation suggest that male mate choice in species with ornamented males may play a large role in the maintenance of species boundaries, contrary to traditional expectations. Male behaviour, including both intra- and intersexual interactions, contributed to over half of the observed behavioural isolation for the focal species pair, with male mate choice alone contributing at least as much as female mate choice. These results suggest that male mate choice may go beyond assessment of female fecundity, even in polygynous species with traditional sex roles. Study systems identifying male mate choice could benefit from considering the potential role of male behaviour in behavioural isolation.

Supplementary Material

Supplementary material associated with this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2017.06.005.

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