Original Article

Sexual selection acting on a speciation trait in darters (Percidae: *Etheostoma*)

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Despite theoretical support linking sexual selection for elaborate coloration and behavioral isolation, few empirical examples clearly demonstrate that the sexually dichromatic signals responsible for behavioral isolation between species are, or have been, subject to sexual selection within species. The present study investigates sexual selection on male nuptial coloration in *Etheostoma barrenense*, a darter species for which male color has been shown in a previous study to contribute to behavioral isolation from a sympatric congener. Through the use of motorized model fish, we demonstrate that female *E. barrenense* discriminate between the orange and red body hues of conspecific males and exhibit an association preference for orange over red models. Combined with the results from a previous study, these data provide evidence for a link between sexual selection and behavioral isolation and by extension for a role of sexual selection in speciation. A post hoc analysis of the colors modeled in darter visual space suggests that quantifying signal values as they are perceived, rather than expressed, provide a promising way of linking sexual selection and behavioral isolation.

Key words: **association preference, behavioral isolation, female choice, male nuptial color, model.**

INTRODUCTION

Sexual selection is thought to facilitate speciation most commonly via the coevolution of male signaling traits and female receiver preferences (Fisher 1930; Lande 1981; West-Eberhard 1983). The result is behavioral isolation, a reproductive barrier characterized by a reduction in courting or mating behavior between individuals of divergent lineages (Coyne and Orr 2004). If sexual selection drives speciation in this way, then females of 2 sympatric species would govern species boundaries by preferring species-specific male traits that are, or have been, subject to sexual selection via female choice within species (Ryan and Rand 1993; Boake et al. 1997; Panhuis et al. 2001).

Although theoretically supported, direct empirical support for speciation by sexual selection is surprisingly weak (Kirkpatrick and Ravigne 2002; Ritchie 2007). The most highly cited evidence for speciation by sexual selection is indirect, demonstrating a positive relationship between a surrogate measure of sexual selection and species richness (Barraclough et al. 1995; Mitra et al. 1996; Møller and Cuervo 1998; Arnqvist et al. 2000; Boughman 2001; Katzourakis et al. 2001; Boughman et al. 2005; Seddon et al. 2008; Kraaijeveld et al. 2011; but see Gage et al. 2002; Morrow et al.

2003; Isaac et al. 2005). Sexual dichromatism in particular is often used as evidence for sexual selection. However, though coloration in many taxa is thought to have arisen by sexual selection (Andersson 1994), the role of these colorful traits in behavioral isolation is less well established (Williams and Mendelson 2011).

Few empirical examples explicitly link sexual selection for elaborate coloration with behavioral isolation by demonstrating that a sexually dichromatic trait contributing to behavioral isolation between species is or has been subject to sexual selection within species. The most convincing studies manipulate color as the sole independent variable and demonstrate that 1) individuals choose conspecific over heterospecific coloration and 2) prefer particular color variants within species. Females of the butterfly species *Pieris occidentalis* discriminate between conspecific males and heterospecific males belonging to the closely related sympatric species *Pieris protodice*, based on melanization patterns of the wings (Wiernasz and Kingsolver 1992). An intraspecific study on *P. occidentalis* showed that females prefer conspecific males with more melanization over those with a smaller surface area of melanin (Wiernasz 1989). Therefore, wing melanization appears to be a sexually selected trait that also maintains behavioral isolation between *P. occidentalis* and *P. protodice*. In the African cichlid *Pundamilia nyererei*, females prefer red conspecific males over blue *Pundamilia pundamilia* males (Seehausen and van Alphen 1998); in addition, these females

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prefer redder males when choosing between a conspecific pair of mates (Maan et al. 2004, 2010). Therefore, the 2 *Pundamilia* species provide another example of how sexual selection could influence speciation via behavioral isolation based on female preference for the same color trait; however, these studies did not isolate color from behavior, so it remains unclear whether color alone can explain female preferences.

Here, we test for empirical evidence of sexual selection on male nuptial color in the darter fish *Etheostoma barrenense*. Darters (Percidae: *Etheostoma*) appear to exemplify the role of sexual selection in speciation. The genus is highly diverse, each species has a unique suite of secondary sexual traits (typically male nuptial color) (Kuehne and Barbour 1983; Page 1983), and behavioral isolation evolves faster than many postmating barriers between allopatric species (Mendelson 2003; Mendelson et al. 2007). Male *E. barrenense* exhibit a vivid orange-red body color (see Figure 1c), whereas males of a close sympatric congener, *Etheostoma zonale*, exhibit a primarily green body color (Williams and Mendelson 2011). These sympatric species are thought to have diverged in allopatry from a common ancestor approximately 6.5 million years ago (based on cytochrome b analysis; Mendelson TC, unpublished data). Although they are not sister species, they represent one of the most closely related pairs of darter species that co-occur without hybridizing in nature (Keck and Near 2009) and whose males exhibit notably distinct body coloration (Gumm and Mendelson 2011; Gumm et al. 2011; Williams and Mendelson 2011). A previous study showed that female *E. barrenense* prefer model males painted to spectrally match the average conspecific body color (orange-red) over green models, the latter spectrally matching male *E. zonale*. In contrast, female *E. zonale* prefer the conspecific-like (green) models, indicating that male nuptial coloration contributes to behavioral isolation in this species pair (Williams and Mendelson 2011).

We now test whether female or male *E. barrenense* discriminate between shorter or longer wavelength orange-red coloration of conspecific male model stimuli, based on variation in body hue measured in a natural population (Gumm and Mendelson 2011). As in most darter species, male *E. barrenense* vary in body hue, but whether females exhibit directional or stabilizing preferences for these hues have not been tested in this or any other darter species. We utilize motorized painted models that allow the isolation of body color (i.e., orange vs. red) as the variable of interest while controlling for confounding variables (e.g., stimulus behavior, shape, and size). We ask whether intraspecific sexual selection acts on a trait (orange-red body coloration) that contributes to behavioral isolation from a sympatric congener (i.e., a "speciation phenotype," sensu Shaw and Mullen 2011). In addition, we use data on the visual system of darters to model the paint colors in the visual space of *E. barrenense* and explore whether differences between stimuli in visual space can predict the strength of behavioral response. As such, we begin to explore a continuum of trait variation within and between species, in order to address the relationship between female preferences and divergence in color signals.

METHODS

Collection and housing

Adult *E. barrenense* were collected from the East Fork of the Barren River in Monroe Co., Kentucky and Line Creek in Clay Co., Tennessee. These streams are both tributaries of the Barren River and locations we have used for previous studies of *E. barrenense*. Permission for the collection and use of these species in behavioral
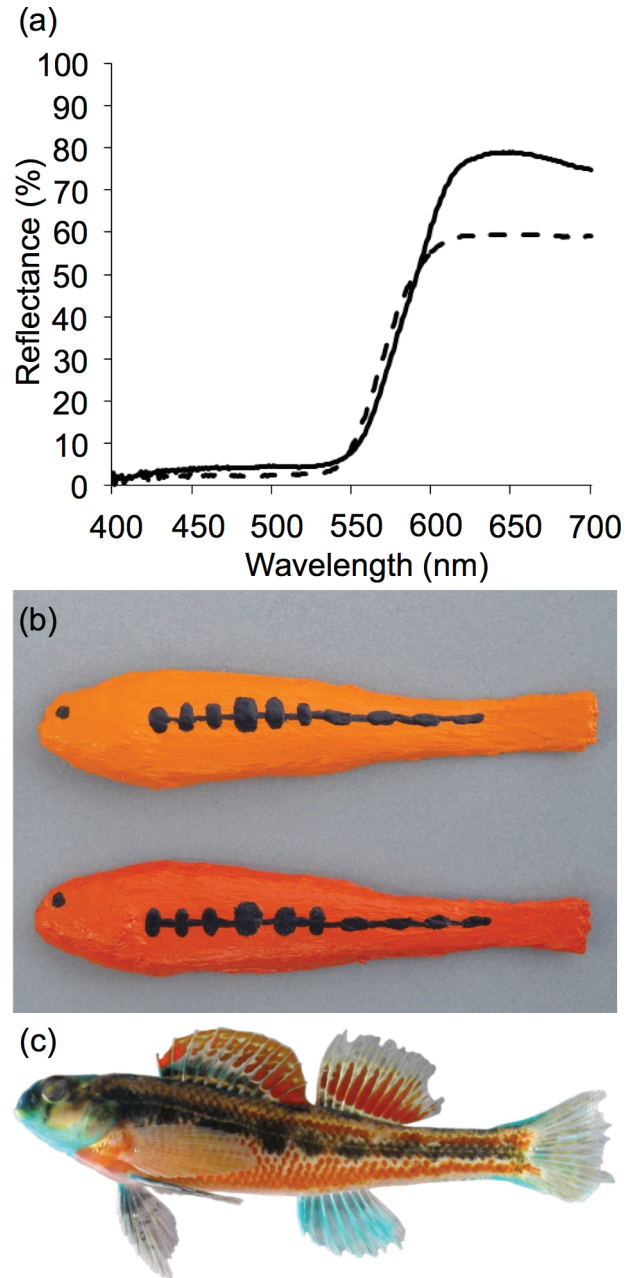
Figure 1

Model coloration. (a) Average reflectance spectra of the orange-painted (dashed line) and red-painted (solid line) models whose hues (wavelength at the reflectance midpoint) differ by 14 nm. (b) Urethane models were painted to mimic the average hue (top; $\lambda_{R50} = 570$ nm) and an extreme hue (bottom; $\lambda_{R50} = 584$ nm) of natural male *Etheostoma barrenense* body coloration. (c) A photograph of a wild caught male *E. barrenense* is shown for comparison.

experiments was granted by the Kentucky Department of Fish and Wildlife Resources and the Tennessee Wildlife Resources Agency. Fish were collected during March and April 2010 and 2011 and were used for study during the 2011 breeding season (approximately 6 April–19 May 2011). Fish were housed at the University of Maryland, Baltimore County in a recirculating aquarium system (Aquatic Habitats, Inc., Apopka, FL) that replicates the natural habitat (water temperature, conductivity, and pH) and is exposed to local natural photoperiod. The fish were separated by sex and

placed in 10-L tanks containing gravel, where they were fed a diet of live black worms and frozen blood worms. All individuals used for testing were fertile adults as indicated by highly colored males and gravid females. Experimental procedures were approved by the University of Maryland, Baltimore County Animal Welfare Assurance (A3784-01) and Institutional Animal Care and Use Committee.

Model preparation

Painted polyurethane models were used to test female preference for color. Models can be painted so that coloration is the single variable of interest while controlling other variables that may be confounded in live animals, for example, behavioral displays, activity levels, and body size or shape. To create the models, a large (standard length = 56 mm), individual adult *E. barrenense* male was euthanized in MS-222 and fixed in 10% formalin. The body was used to create plaster molds for the casting of 20 polyurethane models, as in Williams and Mendelson (2011). Except for the caudal fin, no other fins were present in the models. Male darters raise and lower their dorsal fins while displaying to males and other females, and a static, erect dorsal fin is typically observed only during sustained male contests (personal observation) and therefore may be perceived as a threat. In addition, fin colors in darters often differ from body coloration and are thought to be driven by independent selective pressures (Gumm and Mendelson 2011).

Two hues of paint were chosen based on their spectral similarity to naturally occurring variation in male body coloration in this species (Gumm and Mendelson 2011; Williams and Mendelson 2011). Each model was hand painted. Model color was measured using reflectance spectrometry (Ocean Optics HR2000+, SpectraSuite, Ocean Optics) in 3 locations: head, body, and tail. These 30 measurements per color (10 models per color) were used to generate an average spectral curve for each of the 2 paints (Figure 1a). The average λ_{R50}, or reflectance midpoint, of each step curve is considered by many as an approximation of hue (Hofmann et al. 2006; Kiere et al. 2009; Prager and Andersson 2010; Gumm and Mendelson 2011; Gumm et al. 2011) and was calculated for each color (Figure 1a). "Orange" models had a λ_{R50} of 570 ± 1 nm (standard deviation [SD]), similar to freshly caught wild males whose average λ_{R50} was 566 ± 8 nm (SD) (Gumm and Mendelson 2011). "Red" models had a λ_{R50} of 584 ± 2 nm (SD) and were representative of a naturally occurring extreme hue found on the bodies of male *E. barrenense*. An estimate of brightness was determined by calculating the approximate area under the spectral curve. Relative brightness measurements (in reflectance intensity) were as follows: orange = 8066 and red = 9842. In addition to the red and orange body paints, black paint was stenciled onto each model to add the blotching pattern along the lateral line, characteristic of *E. barrenense*, and to simulate eyes (Figure 1b). Although the use of a stencil minimized large differences in black area between each model, minor variation was expected and justified the use of multiple models to avoid pseudoreplication of stimuli. Although simplistic, similar models were successfully used to test behavior in the focal species (Williams and Mendelson 2011, 2013) and in other fishes (e.g., Speares et al. 2007, round gobies; Baube 2008, three-spined stickleback; Ochi and Awata 2009, African cichlids; Dzieweczynski and Leopard 2010, Siamese fighting fish). The painted models were affixed to thin rods so they could be interchangeably attached to a pair of stepper motors. During each experimental trial, the stepper motors were controlled by a computer program designed by T.H. Williams through GadgetMaster Script Editor v.1.2 software

that communicated to a Gadgetmaster™ driver (LightMachinery Inc., Nepean, Ontario, Canada). The program instructed a pair of model fish to pivot back-and-forth in unison, to control for stimulus behavior (Williams and Mendelson 2011). Although behavioral displays as mating signals are unstudied in this system, the use of motorized models allowed us to control for possible confounding effects of using live males. We animated both models with the same quivering motion that characterizes courtship behaviors of male *E. barrenense*.

Dichotomous choice trials

Preference for orange versus red coloration was tested in both males and females in a series of dichotomous choice trials. Dichotomous choice trials have been used in several species of darters (Fuller 2003; O'Rourke and Mendelson 2010; Williams and Mendelson 2010, 2011, 2013) and have been demonstrated as a reliable proxy for mate choice in many other species of fish (Aspbury and Basolo 2002; Lehtonen and Lindström 2008; Jeswiet and Godin 2011). Darters are not shoaling fishes (Page 1983; Etnier and Starnes 1993), strengthening the validity of association preference tests for estimating mate choice. This protocol also allowed the quantification of "glass jabbing" behavior (the amount of time each test fish spent nudging the partition glass with its snout), which may indicate the fishes' interest in approaching a stimulus. The focal species have previously demonstrated significantly higher levels of glass jabbing behavior toward the same stimuli for which they demonstrate the higher association preference (Williams and Mendelson 2011, 2013). The dichotomous choice setup consisted of 3 aquaria, with a 37-L tank placed between a pair of 9.5-L tanks. The middle tank was visually sectioned into 3 zones: the left and right association zones (each 5 cm in length) and a central neutral zone (40 cm in length). Each tank contained the same gravel depth and was filled to the same height with fresh aquarium–housing system water prior to each trial. Full spectrum light illuminated the triad of tanks via a full spectrum lamp (Coralife® F/W T-5 Aqualight, 21 W Colormax™ bulb, 21 W 6700K bulb) and supplementary incandescent lighting (GE Crystal Clear, A19, 100 W).

Prior to each trial, a pair of models were selected (1 orange and 1 red model that were not previously paired together), fastened to the pair of stepper motors, and submerged into the pair of 9.5-L tanks so that one stimulus was visible on either end of the central 37-L tank. An individual test fish was placed in the center of the central tank and allowed to acclimate. Once free-swimming behavior was observed, the models were mobilized to draw the test subject's attention to the association zones. The trial began after the fish visited both the left and right association zones and presumably saw both the orange and red models. The location of the test fish throughout a 20-min trial, specifically the time spent occupying the right and left association zones, was documented using the program JWatcher™. The amount of time each test fish spent glass jabbing in either of the association zones was also documented. Glass jabbing was scored as a duration, rather than events, as the behavior occurs rapidly and consecutively in clearly defined bouts. Although association time and glass jabbing by females may indicate an interest in mating, these same measures observed in males may correspond to aggression. Each trial tested a unique individual in the presence of a unique pair of model stimuli. The presentation of the model colors on the right and left sides of the test tank was determined pseudorandomly between replicates, such that 50% of the replicates presented "orange" on the left and "red" on the right, and vice versa for the other 50%, to eliminate possible effects of

side bias. Nineteen female and 21 male fish were assessed for association preference.

Analysis

Each test fish acclimated to the experimental apparatus appropriately and visited at least 1 preference zone during the trial; thus, all test fish were deemed "active" and included in analysis. Data on association times were tested for normality using the Shapiro–Wilk test. A square-root transformation on the data representing preference zone occupation by females exhibited a normal distribution; thus, a paired t-test was used to analyze the data set. After all attempts at transformation on the other data sets in the study, the assumptions of parametric statistics were not met and nonparametric statistics were used for all remaining analyses, and time spent with stimuli was assessed using Wilcoxon sign ranks tests.

The strength of preference (SOP) was measured for each individual test fish as

$$\text{SOP} = \frac{T_O - T_R}{T_O + T_R} \tag{1}$$

where T_O and T_R represent time spent in the preference zones adjacent to the orange and red models, respectively. This index of preference allows for comparisons across different treatment groups. SOP was compared between the sexes using a Mann–Whitney U test. All statistics were performed in IBM SPSS Statistics v. 20 (Chicago, IL).

Visual modeling

Visual sensitivity to the orange- and red-painted models was determined using a visual modeling template (courtesy of K. Carleton and B. Dalton) in Microsoft Office Excel. The template allowed for the calculation of quantal catch (Q) for each photoreceptor type (i) according to Equation 2 (e.g., Endler and Mielke 2005; Stevens et al. 2009; Dalton et al. 2010):

$$Q_i = K_i \int R_i(\lambda)L(\lambda)S(\lambda)I(\lambda)d\lambda \tag{2}$$

R_i represents the sensitivity of photoreceptor i taken as the normalized absorbance of its specific visual pigment, based on standard visual pigment templates (Govardovskii et al. 2000). Darters have a medium-wavelength sensitive (MWS) and a long-wavelength sensitive (LWS) cone type, each associated with A2 visual pigments (Gumm et al. 2011). According to preliminary microspectrophotometric data from 8 female *E. barrenense*, this species expresses LWS double cones with an average peak absorbance at 600.65 ± 5.30 nm (SD) and MWS single cones with an average peak absorbance at 511.89 ± 4.72 nm (SD) (Gumm JM, Mendelson TC, unpublished data). These data were used to generate spectra using Govardovskii A2 templates for use as R_i in the visual modeling (Govardovskii et al. 2000). Quantal catch also depends on the transmittance spectra of the cornea and lens (L); data on cornea and lens transmittance were taken from sister species *Etheostoma rafinesquei* (Gumm JM, Mendelson TC, unpublished data), as data for *E. barrenense* were not available. Surface reflectance of the stimuli (S) was determined using the average reflectance spectra of the orange- and red-painted models (see above). Illumination of the background (I) was previously calculated by normalizing environmental light spectra for the same testing apparatus used in the present study (Williams and Mendelson 2011). The von Kries factor (K_i) for each receptor was based on the von Kries color constancy model, which assumes independent adaptation to the background (Kelber et al. 2003).

The von Kries factor is described by Equation 3 (e.g., Vorobyev and Osorio 1998; Dalton et al. 2010):

$$K_i = \frac{1}{\int R_i(\lambda)L(\lambda)I(\lambda)d\lambda} \tag{3}$$

where $I(\lambda)$ is the background radiance.

RESULTS

Female choice

Female *E. barrenense* spent significantly more time occupying the preference zone adjacent to the orange model, similar to the average male hue, compared with the red model ($X \pm \text{SD} = 37.57 \pm 24.10\%$ vs. $15.94 \pm 20.23\%$ of total trial time, $t_{18} = 2.461$, $P = 0.024$) (Figure 2a). Similarly, females spent more time performing glass jabbing behavior toward the orange model than the red model, but this difference was not statistically significant ($13.30 \pm 17.60\%$ vs. $7.49 \pm 16.63\%$ of total trial time, $Z_{18} = -1.350$, $P = 0.177$).

Male choice

Male fish also occupied the preference zone adjacent to the orange model ($29.71 \pm 35.19\%$) longer than that of the red model ($16.88 \pm 23.89\%$) but not significantly so ($Z_{20} = -1.167$, $P = 0.243$) (Figure 2b). In addition, glass jabbing behavior was directed more toward the orange model ($12.43 \pm 24.74\%$) than the red model ($4.99 \pm 12.88\%$), but this difference was not statistically significant ($Z_{20} = -1.153$, $P = 0.249$).

Between-sex comparisons

SOP ranged from -1, indicating a strong preference for the red models, to 1, indicating a strong preference for orange models for both sexes. Females had a stronger average SOP for orange models (0.37 ± 0.71) than did males (0.11 ± 0.84), but the difference in SOP between males and females was not statistically significant ($Z_{18} = -1.160$, $P = 0.246$).

Visual modeling and post hoc analysis

The normalized quantal catch values for the orange model were $Q_{MWS} = 23.66\%$ and $Q_{LWS} = 76.34\%$ and for the red model were $Q_{MWS} = 21.41\%$ and $Q_{LWS} = 78.59\%$ (Figure 3). Therefore, the difference in quantal catch between the 2 stimuli, measured as a ratio of Q_{LWS} to Q_{MWS}, is $(78.59/21.41) - (76.34/23.66) = 0.44$. This metric estimates how different the paired stimuli appear to female *E. barrenense*, and we can use this metric to compare an individual's SOP for the preferred stimulus with the degree to which 2 stimuli differ in visual space. In this study, the difference in quantal catch of the 2 stimuli is 0.44 and the SOP for orange over red is SOP = 0.37. We can compare this relationship with that of the previous study that tested female preference for red (conspecific) over green (heterospecific) models. Substituting reflectance spectra of the red and green models into the template above yielded $Q_{MWS} = 27.01\%$ and $Q_{LWS} = 72.99\%$ for the *E. barrenense* type model (orange-red) and $Q_{MWS} = 58.05\%$ and $Q_{LWS} = 41.95\%$ for the *E. zonale* type model (green). Thus, the average difference in Q_{LWS}/Q_{MWS} between the 2 stimuli for that experiment was $(72.99/27.01) - (41.95/58.05) = 1.98$. The average SOP for the conspecific-type color over the heterospecific was 0.70, nearly twice as strong as the preference for orange over red. Thus, comparing the difference between model stimuli in visual space and the SOP
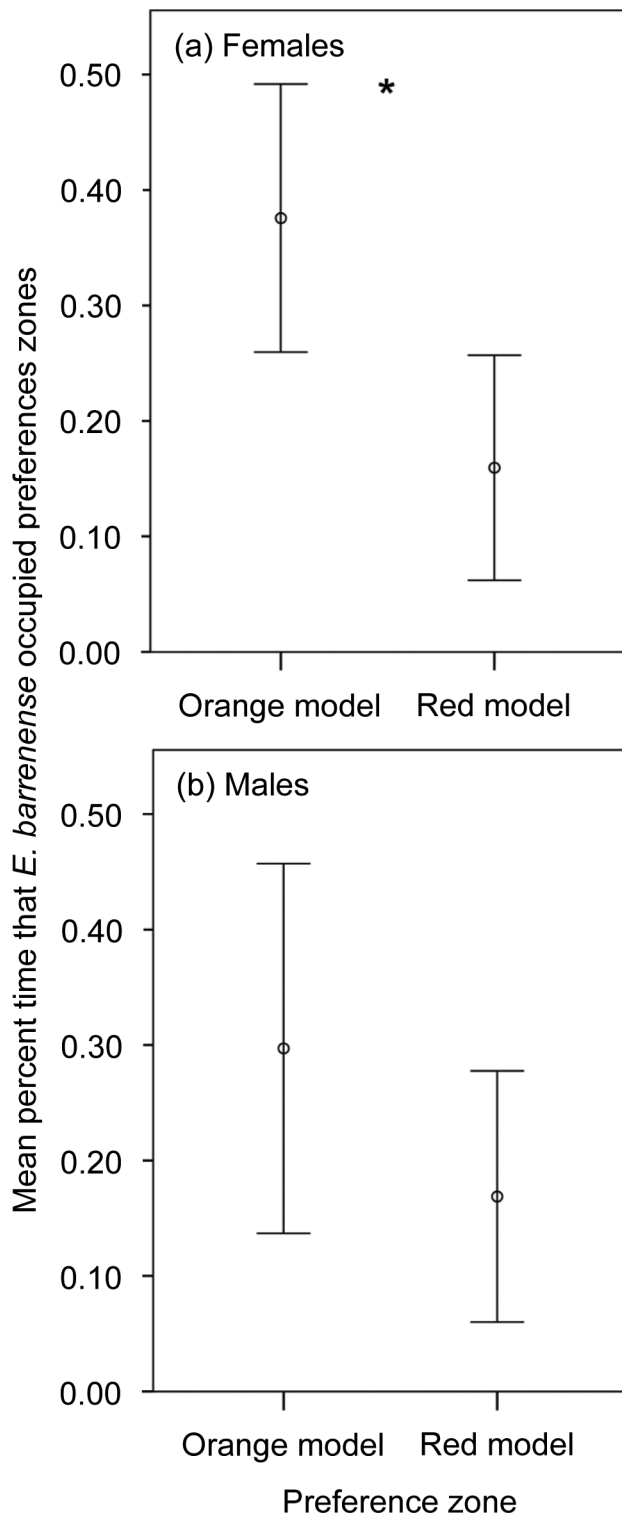
Figure 2
Preference for orange versus red models exhibited by female (a, *n* = 19) and male (b, *n* = 21) *Etheostoma barrenense*. Dots indicate the mean percent of trial time that fish spent in a 5-cm preference zone adjacent to either model type. Whiskers represent 95% confidence intervals and the asterisk notes a significant difference.

for the preferred stimulus allows us to examine trait variation in a way that links preferences for color within species and preferences for color between species.

DISCUSSION

Female *E. barrenense* spent more time associating with the orange models than the red models, suggesting females in this species exhibit a preference for a particular color variant exhibited by wild males (Williams and Mendelson 2011). This finding supports the hypothesis that sexual selection by female choice can drive the evolution of male nuptial coloration in *E. barrenense*. This finding alone is not a conclusive or exclusive test of the hypothesis of sexual selection on male color and may benefit from supplementary tests (i.e., directly measuring variation in hue as it correlates to fitness) or the elimination of alternative explanations (e.g., ecological selection). Nonetheless, demonstrating female preference for a specific trait value within a species suggests female preference can exert selection on male nuptial coloration in darters and provides a link between sexual selection and speciation, as the trait has been shown in a previous study to contribute to maintenance of behavioral isolation from a closely related sympatric species (Williams and Mendelson 2010, 2011).

Of the 2 sexes, only females exhibited a statistically significant preference for the orange models. This pattern conforms to the Darwinian theory of sexual selection in a sexually dimorphic species, whereby cryptic females show a preference for elaborate males (Darwin 1871). Previous work demonstrated that females prefer color and pattern elements of conspecific males over those of a sympatric heterospecific species (Williams and Mendelson 2011). Females of a different darter species, *Etheostoma nigripinne*, also have been shown to strongly prefer conspecific males over heterospecifics, whereas males did not exhibit a significant preference for conspecific females (O'Rourke and Mendelson 2010). However, the present study is the first to demonstrate a preference in females for a particular male color variant within a single darter species. By using motorized models and isolating color as the only variable of interest, we also can rule out additional traits such as behavior and body shape as confounding factors.

Males did not exhibit a significant preference for either model color but did show a general trend toward preferentially associating with the orange model. Although males did not statistically distinguish between the orange and red stimuli in the present study, they have been shown to associate preferentially with models painted to resemble conspecifics over heterospecific models (Williams and Mendelson 2013). One hypothesis is that substantial variation in male nuptial coloration, that is, on the scale of interspecific variation, has informational value to other males (e.g., whether a sexual competitor is present). Indeed, a tendency in males to attack a homotypic color and ignore a heterotypic color in African cichlids (Dijkstra et al. 2005; Dijkstra 2006; Dijkstra, Seehausen, et al. 2007; Pauers et al. 2008) may contribute to the rapid and widespread diversification of this lineage (Seehausen and Schluter 2004). On an intraspecific level, however, variation in male nuptial coloration may not be informative (e.g., an orange male may not indicate more or less of a sexual competitor than a red male). Alternatively, our study may lack statistical power, and the general trend toward male association preference for the orange model may indicate elevated aggression toward orange hue and suggest a role for male–male competition. Variation in male nuptial coloration within *P. nyererei* correlates with intraspecific social rank as redder males often chase and displace less red males (Dijkstra, Hekman, et al. 2007). Studies of cichlid fish, therefore, suggest a role for elaborate male coloration in identifying conspecific rivals and possibly for intrasexual selection in a highly diverse and sexually dichromatic fish lineage.
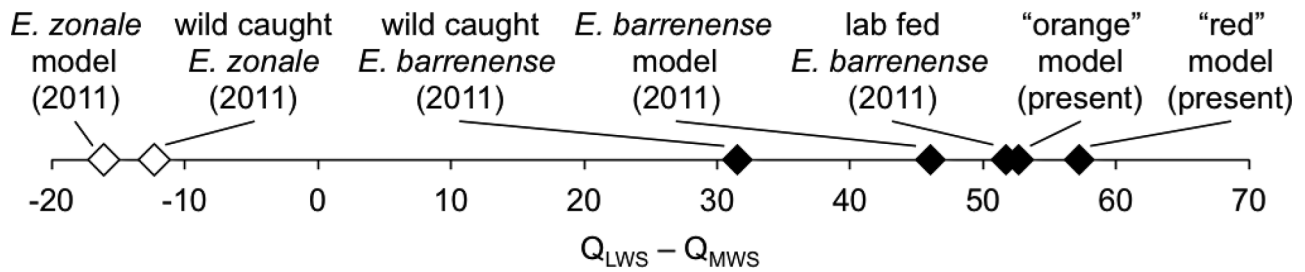
Figure 3

Estimates of quantal catch ($Q_{LWS} − Q_{MWS}$) for female *Etheostoma barrenense* for model colors. Values for natural fish colors and "green" *Etheostoma zonale* stimuli are included for comparison (Williams and Mendelson 2011).

However, from the perspective of the present study on sexual selection within a single darter species, results favor the hypothesis that females have a greater role in driving selection on male nuptial color within species.

In the dichotomous choice trials, test fish were subject to pairs of stimuli that varied by 14 nm in hue, measured as the midpoint of the step-shaped reflectance curve of the paints used here and typical of carotenoid pigments (Hofmann et al. 2006). The preferred hue, orange, had a spectral location value that was very close to the mean spectral location value of freshly caught, wild male *E. barrenense*. The red models had a spectral location value that was occasionally found in males; however, it was not close to the population mean but rather representative of an extreme trait value (Gumm and Mendelson 2011) (Figure 3). Therefore, based on information from hue, we might speculate that male body coloration is subject to stabilizing, rather than directional selection; however, additional studies with a wider range of color options will be necessary to adequately test that hypothesis.

Interestingly, the hue of the less preferred red models better matched the hue of laboratory-fed males (wild caught males whose color was measured 1 year postcaptivity; average λ_{R50} was 584 ± 3 nm) (Williams and Mendelson 2011). These lab males had been fed frozen blood worms (Hikari®) fortified with carotene on a daily basis, which may have enhanced the natural body hue toward red as has been demonstrated in other fish species (e.g., Grether et al. 1999; Clotfelter et al. 2007). It is, therefore, curious that the red models were not preferred over orange, because the redder hue was exhibited by presumably more well-fed males, and carotenoid-based colors of longer wavelength serve as an indicator of high-quality mates in other species. Female house finches prefer to mate with red males over orange or yellow males (Hill 1990, 1991, 1999) and these preferences appear to have an impact on reproductive success (McGraw et al. 2001); however, carotenoid compounds used in house finches (Prager and Andersson 2009) are likely different than those in darters and results may not apply to our study. Male three-spined sticklebacks also vary between orange and red throat coloration, which is highly influenced by carotenoid consumption (McLennan 2006). Coloration is thought to signal mating benefits to females (Milinski and Bakker 1990; Bakker 1993; Candolin 2000; Rick et al. 2011), as high condition females prefer red males, whereas low condition females prefer orange males in association tests (Bakker et al. 1999).

An alternative approach to evaluating color preferences that may explain why *E. barrenense* prefer orange over red is to model the color options in putative visual space (i.e., quantal catch; Figure 3), rather than quantifying hue (λ_{R50}). Measured on a continuum of quantal catch rather than hue, the orange (preferred) models in the present study more closely resemble the quantal catch from the extreme "red" values of live, lab-fed males (Figure 3). Quantal catch from the red (not preferred) models represents an even further extreme that we have not observed in either freshly caught or lab-fed males. Therefore, testing female responses to trait values at both upper and lower extremes of quantal catch values will further improve estimates of the mode of selection acting on male nuptial color (e.g., stabilizing or directional). Regardless, these data suggest females are exerting selection on a trait that contributes to behavioral isolation, and the preference does not appear open ended (Ryan and Keddy-Hector 1992), as the most extreme value of red by both measures, hue (λ_{R50}) and quantal catch, was not preferred.

Another benefit of quantifying color in visual space is that it allows intra- and interspecific variation to be measured along a continuum. One of the main challenges in linking sexual selection with behavioral isolation is that signals of different species can differ in kind, rather than degree, such that signals that form the basis of within-species preferences cannot be measured along the same axis of variation as signals that form the basis of between-species preferences (Ryan and Rand 1993). Animal coloration is a good example. Though in one sense color varies along a single axis (wavelength of light), the pigments that produce colors in different portions of the visible spectrum are categorically and mechanistically distinct, so variation between colors cannot be measured as a single quantitative trait. For example, variation in hue of the orange-red body color of *E. barrenense* should not be measured on the same axis as variation in the green body color of *E. zonale*, because red pigments in darters are carotenoid based (Porter BA, personal communication), whereas green and blue pigments appear to be derived from a biliprotein, as in some other fishes (Goda and Fujii 1995; Yu et al. 2008). Additionally, the reflectance spectra of these different colors are shaped differently, limiting the ways in which they can be quantified as continuous variables.

If color differences are modeled instead according to how they might be perceived, we can quantify variation both within and among species along a single continuum, for example, quantal catch. Results of our analyses reveal a match between the SOP for one signal over another and the degree to which those signals differ in visual space: the more 2 stimuli differed in visual space, the greater the level of attraction toward the preferred stimulus (i.e., the average preference for orange over red in this study was not as strong as the average preference for orange/red over green in the previous study). A sample size of 2 prevents statistical analysis; however, a relationship between difference in visual space and SOP suggests that measuring signal values as they are perceived, rather than expressed, provides a promising avenue for investigating the link between sexual selection and behavioral isolation.

Finally, although orange-red body coloration appears to play a role in both sexual selection in *E. barrenense* and behavioral isolation

between *E. barrenense* and *E. zonale*, other signals may be important in both contexts. The present study utilized motorized model fish to hold the behavior of stimuli constant, isolating body coloration as the sole variable of interest. However, given the discrepancy in brightness between the 2 model paints, we cannot rule out the possibility that color brightness plays a role in association preference, either in isolation or in concert with hue. Additionally, in more natural settings, courting behavior of males could also influence female preference either within or between species (Byers et al. 2010). Cues in other modalities, such as chemical cues, may also be involved in female preferences (Candolin 2003). Thus, traits linking sexual selection and behavioral isolation may be complex, and a comprehensive understanding of these traits and their interactions requires careful experiments that test the role of multiple signals in both contexts.

In sum, the present study provides empirical evidence that a trait contributing to the maintenance of behavioral isolation between species may be subject to sexual selection within species. Species boundaries between *E. barrenense* and *E. zonale* are likely maintained via behavioral isolation, as other reproductive barriers between them are incomplete (Williams TH, Mendelson TC, in review). Male nuptial coloration in this species pair, therefore, may be a critical "speciation phenotype" (Shaw and Mullen 2011), acting as one of the strongest barriers to gene flow. The use of model fish and visual modeling techniques provides a strong framework for further study of these phenotypes in *Etheostoma* or other species of colorful fishes.

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