Changes in sexual signals are greater than changes in ecological traits in a dichromatic group of fishes

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Understanding the mechanisms by which phenotypic divergence occurs is central to speciation research. These mechanisms can be revealed by measuring differences in traits that are subject to different selection pressures; greater influence of different types of selection can be inferred from greater divergence in associated traits. Here, we address the potential roles of natural and sexual selection in promoting phenotypic divergence between species of snubnose darters by comparing differences in body shape, an ecologically relevant trait, and male color, a sexual signal. Body shape was measured using geometric morphometrics, and male color was measured using digital photography and visual system-dependent color values. Differences in male color are larger than differences in body shape across eight allopatric, phylogenetically independent species pairs. While this does not exclude the action of divergent natural selection, our results suggest a relatively more important role for sexual selection in promoting recent divergence in darters. Variation in the relative differences between male color and body shape across species pairs reflects the continuous nature of speciation mechanisms, ranging from ecological speciation to speciation by sexual selection alone.

KEY WORDS: Digital photography, Etheostoma, morphometrics, natural selection, sexual selection, speciation.

Biological diversity is a striking, visible manifestation of evolution, and understanding the mechanisms by which lineages diversify is a central focus of evolutionary research. In particular, work in several animal systems supports a role of divergent, ecologically based selection in the process of speciation (i.e., “ecological speciation”; Hatfield and Schluter 1999; Langerhans et al. 2007; Nosil et al. 2007; for review see Rundle and Nosil 2005; Nosil 2012). During this process, divergence results as populations adapt to different environments, leading to reproductive isolation as a byproduct of local adaptation (Schluter 2001; Gavrilets 2004; Servedio et al. 2011). While ecological speciation is clearly an important driver of diversity in some systems, speciation in other groups may require an additional or alternative explanation. In particular, ecological speciation may not best explain animal radiations characterized by diversity in secondary sexual traits and minimal ecological differences (e.g., crickets, Mendelson and Shaw 2005; jumping spiders, Masta and Maddison 2002; frogs, Boul et al. 2007; for reviews see Prum 2012; Rodriguez et al. 2013). Speciation by sexual selection offers an alternative mechanism of divergence that does not require environmental differences (Lande 1981; Panhuis et al. 2001; Ritchie 2007).

Understanding the relative importance of ecological selection and sexual selection is a central question in speciation research (Marie Curie Speciation Network 2012; Safran et al. 2013). While ecological speciation and speciation by sexual selection are often offered as categorical alternatives, divergence within any given group likely falls along a continuum ranging from “ecological adaptation alone” to “sexual selection alone” (Safran et al. 2013). For example, some models of sensory drive, in which environmental differences influence sexual selection, require an interaction of ecological adaptation and sexual selection (Boughman 2001; Seehousing et al. 2008). A first step toward understanding the relative influence of divergent ecological selection and sexual selection in speciation is to examine differences in traits that correspond to these types of selection (Safran et al. 2012). Greater divergence
in these associated traits indicates a greater, but not necessarily exclusive, influence of one type of selection over another. For example, ecological characters diverge at a slower rate than sexual signals in *Paramormyrops* electric fishes (Arnegard et al. 2010) and several other taxa (Safran et al. 2012), suggesting an important role for sexual selection in divergence.

Sexual signals run the gamut of sensory modalities, with visual mating signals being some of the most obvious and well-studied examples of secondary sexual traits (Andersson 1994; Price 2008). Several species radiations exhibit extensive diversity in visual signals (i.e., Masta and Maddison 2002; Allender et al. 2003; Hoffman et al. 2006; Nicholson et al. 2007; Gum and Mendelson 2011), and visual signals are often used in comparative studies to examine the relative roles of natural and sexual selection in speciation (e.g., Barraclough et al. 1995; reviewed in Ritchie 2007). Yet, few studies, if any, quantitatively test whether divergence in visual sexual signals is greater than in ecological traits across a diverse species radiation. Other studies that quantitatively compare traits do not examine visual signals (Arnegard et al. 2010), or they compare trait differences across closely related taxon pairs rather than across a diverse clade (Safran et al. 2012).

Objectively quantifying differences among visual signals, and color in particular, can be difficult due to their complexity and differences between our visual system and the visual system of the intended receivers. This difficulty has hindered quantitative comparison of color signals to other phenotypic traits. Spectral reflectance measures have been used as a quantitative, objective measure of color (e.g., Leal and Fleishman 2004; Hofmann et al. 2006; Clotfelter et al. 2007; Gum et al. 2011; Gum and Mendelson 2011), but these measurements often require expensive equipment that can be difficult to use in the field. Characterizing a multicomponent signal also may require time-intensive sampling. Color analysis using digital photography, however, measures entire color signals relatively quickly and allows flexibility in analysis (for review see Stevens et al. 2007). And like reflectance spectrometry, postprocessing of digital photographs can allow color signals to be interpreted in the context of the receiver’s visual system (Stevens et al. 2009; Pike 2011; Ligon and McGraw 2013; see Materials and Methods).

Darters are a radiation of small fishes native to North American streams and lakes. In total, approximately 200 species of darters have been described, with centers of diversity in the southeastern United States (Page and Burr 2011). Most darter species are sexually dichromatic, with males expressing nuptial coloration in the breeding season. Many closely related species differ primarily in elaborate male nuptial colors (e.g., Etnier and Bailey 1989; Powers and Mayden 2003, 2007), and these colors appear to be subject to sexual selection. Empirical studies show that females prefer the mean hue of conspecific males (Williams et al. 2013), females in two species prefer the nuptial color of conspecifics over heterospecifics (Williams and Mendelson 2013), and differences in male nuptial color predict male aggression bias across multiple species (M. D. Martin and T. C. Mendelson, unpubl. ms.). Darter colors are produced with carotenoids and other pigments as well as structural elements (Porter et al. 2002; Boone 2011). Although some aspects of color may be influenced by environmental conditions, species-level variation in color patterns is maintained in the laboratory, suggesting a genetic component to these differences (M. D. Martin and T. C. Mendelson, pers. obs.). Nuptial color appears to diverge independently of environmental differences in darters, with no effect of micro- or macrohabitat differences on color divergence among closely related species (Martin and Mendelson 2012). Finally, distantly related darter species with widely divergent coloration are often found syntopically, such that distinct hues and patterns are maintained in a common environment.

Darters also exhibit ecological specialization in some traits as a result of occupying habitats that differ in water depth, water velocity, and substrate size. Mouth location and jaw morphology are typical examples of ecological adaptation in fishes, but this variation is relatively low in darters in comparison to other fish radiations (Carlson and Wainwright 2010). Darters are opportunistic feeders (van Snik Gray et al. 1997; but see Alford and Beckett 2007), and minor differences in feeding morphology are thought to reflect foraging habitat differences rather than prey item differences (Carlson and Wainwright 2010). Body shape variation, however, is more pronounced in darters, and generally corresponds to water velocity and substrate size. Benthic species in fast, rocky riffles exhibit deeper bodies and caudal peduncles, whereas those in slow, sandy habitats exhibit slender bodies (Page and Swofford 1984). Body shape variation is arguably therefore a better measure of ecological adaptation. Because body shape differences appear to characterize more distantly related species, whereas sexual trait differences characterize all species, we hypothesize that sexual selection has a greater influence on phenotypic divergence during the earliest stages of speciation. We empirically test that hypothesis by comparing differences in nuptial color with differences in body shape across closely related species of darters.

**Materials and Method**

**SPECIMEN COLLECTION AND CARE**

Specimens were collected in the spring of 2010, 2011, and 2012 (Table 1). All specimens were used for body shape analysis; specimens collected in 2012 were used in male color analysis. After collection, specimens were transported in aerated coolers to the laboratory in Baltimore, Maryland. Fish were kept in temperature controlled, recirculating aquaria (Aquatic Ecosystems, Apopka, FL) under a 12-h dark:12-h light cycle. We fed the
Table 1. Specimen collection localities.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Year</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Etheostoma baileyi</em></td>
<td>Clear Crk., Rockcastle Co., KY</td>
<td>37.477515</td>
<td>−84.26038</td>
<td>2012</td>
<td>4 (4)</td>
</tr>
<tr>
<td><em>Etheostoma barrenense</em></td>
<td>East Fork Barren R., Monroe Co., KY</td>
<td>36.745659</td>
<td>−85.6968</td>
<td>2010</td>
<td>5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>10 (10)</td>
</tr>
<tr>
<td><em>Etheostoma cervus</em></td>
<td>Clarks Crk., Chester Co., TN</td>
<td>35.49792</td>
<td>−88.58793</td>
<td>2010</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>15 (15)</td>
</tr>
<tr>
<td><em>Etheostoma coosae</em></td>
<td>Conasauga R., Polk Co., TN</td>
<td>35.011264</td>
<td>−84.725196</td>
<td>2010</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>19 (19)</td>
</tr>
<tr>
<td><em>Etheostoma etnieri</em></td>
<td>Cherry Crk., White Co., TN</td>
<td>36.004708</td>
<td>−85.432827</td>
<td>2011</td>
<td>9</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>10 (8)</td>
</tr>
<tr>
<td><em>Etheostoma flavum</em></td>
<td>Wartrace Crk., Bedford Co., TN</td>
<td>35.58812</td>
<td>−86.339457</td>
<td>2011</td>
<td>9</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>10 (10)</td>
</tr>
<tr>
<td><em>Etheostoma inscriptum</em></td>
<td>Little Eastatoe Crk., Pickens Co., SC</td>
<td>34.96028</td>
<td>−82.82095</td>
<td>2010</td>
<td>19</td>
</tr>
<tr>
<td><em>Etheostoma lyncium</em></td>
<td>Big Spring Crk., Marshall Co., MS</td>
<td>34.71106</td>
<td>−89.3938</td>
<td>2011</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Fishing Crk., Pulaski Co., KY</td>
<td>37.16401</td>
<td>−84.707558</td>
<td>2010</td>
<td>7</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>17 (17)</td>
</tr>
<tr>
<td><em>Etheostoma pyrrhogaster</em></td>
<td>Thompson Crk., Weakley Co., TN</td>
<td>36.29446</td>
<td>−88.57148</td>
<td>2010</td>
<td>11 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>9</td>
</tr>
<tr>
<td><em>Etheostoma ramseyi</em></td>
<td>Little Schultz Crk., Bibb Co., AL</td>
<td>33.039644</td>
<td>−87.123005</td>
<td>2010</td>
<td>5 (18)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2011</td>
<td>10 (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>18 (16)</td>
</tr>
<tr>
<td><em>Etheostoma raneyi</em></td>
<td>Big Spring Crk., Marshall Co., MS</td>
<td>34.71106</td>
<td>−89.3938</td>
<td>2010</td>
<td>14 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2011</td>
<td>11 (11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>11 (11)</td>
</tr>
<tr>
<td><em>Etheostoma rapestre</em></td>
<td>Sipsey Crk., Lamar Co., AL</td>
<td>34.062366</td>
<td>−88.145206</td>
<td>2011</td>
<td>8</td>
</tr>
<tr>
<td><em>Etheostoma tallapoosae</em></td>
<td>Buck Crk., Clay Co., AL</td>
<td>33.259468</td>
<td>−85.772803</td>
<td>2010</td>
<td>14 (13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>2011</td>
<td>10 (16)</td>
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<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>18 (16)</td>
</tr>
<tr>
<td><em>Etheostoma thalassinum</em></td>
<td>Middle Saluda R., Greenville Co., SC</td>
<td>35.07850</td>
<td>−82.53641</td>
<td>2010</td>
<td>18 (15)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>17 (16)</td>
</tr>
<tr>
<td><em>Etheostoma zonistium</em></td>
<td>Lick Crk., McNairy Co., TN</td>
<td>35.06908</td>
<td>−88.44284</td>
<td>2010</td>
<td>15</td>
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<td>10</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2012</td>
<td>4 (4)</td>
</tr>
</tbody>
</table>

Sample sizes for each collection year are also included. Sample sizes of color analyses given in parentheses. Overall, 434 and 192 specimens were analyzed for body shape and male color, respectively.

Fish live blackworms twice daily. Within 1 month of capture, fish were euthanized using an overdose of tricaine methanesulfonate (MS-222; Acros Organics, Geel, Belgium). Use of MS-222 can decrease overall color reflectance in some fishes but not color value (i.e., hue; Gray et al. 2011), which is our measure of interest. Specimens were briefly placed (approximately 2 min) in 10% formalin with fins extended prior to photographing. Formalin preservation can result in body arching and thereby affect body shape measurements (Arnegard et al. 2010). Our control for this potential influence is described below.

**IMAGE ACQUISITION AND POST PROCESSING**

Male color was analyzed using calibrated digital images (for review see Stevens et al. 2007; for detailed methodology see Ciccotto et al. 2014). All specimens were photographed under incandescent lighting that provides a more uniform light spectrum than fluorescent lighting. Photographs were taken using a PowerShot A650 IS (Canon USA, Inc., Lake Success, NY) in the RAW file format using the CHDK firmware (available at: http://chdk.wikia.com/wiki/Downloads). The RAW file format is a “lossless” file format, unlike JPEG, which uses data compression to minimize file size and may distort the
camera data (Stevens et al. 2007). Camera settings were kept constant across specimens and the internal white balance information from the camera was ignored to minimize variation across images. RAW files were converted to tagged image file format (TIFF) using “dcraw” (version 9.06, Dave Coffin, available at http://www.cybercom.net/~dcoffin/dcraw/). Each image included a Mini ColorChecker (X-rite, Inc., Grand Rapids, MI) allowing for color calibration in Adobe Photoshop (version 10.0.1, Adobe Systems, Inc., San Jose, CA) using the inCamera plug-in (version 4.5, PictoColor Software, Burnsville, MN). An additional calibration step, linearization, was implemented in MATLAB (version 7.13.0.564, The MathWorks, Inc., Natick, MA). Linearization ensures that the camera sensors are responding accurately across brightness levels and that all color channels (i.e., red, green, and blue [RGB]) are equalized (i.e., green is not overrepresented in the photograph due to an in-camera bias). Camera response to achromatic stimuli was assessed using RGB color values for each of the achromatic color patches from the ColorChecker chart. Average RGB values were calculated using ImageJ (version 1.44p; Schneider et al. 2012) and photo corrections were undertaken using the “getlincam” and “lincam” MATLAB functions written by Westland and Ripamonti (2004). Example code can be found in the Supporting Information Appendix S1. The resulting images provide a representational measure of the entire color signal (i.e., complete lateral view of the male darter).

CAMERA SENSOR CHARACTERIZATION

To convert RGB values into darter specific color values, we needed to first estimate the camera sensitivity. Camera sensitivity functions for our Canon Powershot A650IS were estimated using methods outlined by Pike (2011). Function estimation requires (1) measuring a set of color standards using spectrophotometry, and (2) measuring camera response values (i.e., R, G, and B values) to these same color standards. Reflectance spectra for each of the 24 color patches in the ColorChecker chart were measured against a Spectralon® white standard using a HR2000+ spectrometer (Ocean Optics, Dunedin, FL) under illumination from a halogen light source (Fostec, Auburn, NY). Spectra were averaged over 100 measurements and a 30 nm boxcar smoothing window was applied. Irradiance measurements of the photography setup were taken using the same device. The products of these reflectance spectra and irradiance spectra comprise the matrix C (see Pike 2011) used in camera sensitivity function estimation. Camera response values for each of the 24 color patches in the ColorChecker chart were averaged and used to estimate generalized MWS and LWS cone sensitivities applicable to the darter species examined here. Cone sensitivity functions were estimated using Govardovskii templates (Govardovskii et al. 2000).

DARTER VISUAL SYSTEM CHARACTERIZATION

Converting camera response values to darter-specific values also requires an understanding of the darter visual system. Previous work has characterized the spectral sensitivity of retinal cone cells of several darter species. Two color sensitive cone types have been detected by microspectrophotometry in several darter species: a MWS (middle wavelength sensitive) cone and a LWS (long wavelength sensitive) cone (Gumm et al. 2012). The absence of a SWS cone has been confirmed by qRT-PCR (J. Gumm, unpubl. data). Peak absorbance data for Etheostoma baileyi, E. coosae, E. ramseyi, and E. tallapoosae were averaged and used to estimate generalized MWS and LWS cone sensitivities applicable to the darter species examined here. Cone sensitivity functions were estimated using Govardovskii templates (Govardovskii et al. 2000).

MAPPING TO DARTER COLOR SPACE

A mapping function to convert RGB color values to darters’ MWS and LWS values was estimated using methods outlined by Westland and Ripamonti (2004) and Stevens et al. (2007) using custom MATLAB scripts (Appendix S3). Camera sensitivity and darter cone sensitivity functions, along with reflectance spectra from the Floral Reflectance Database (accessible at http://www.reflectance.co.uk/, Arnold et al. 2010) were used to estimate camera and darter response values for 1910 naturally occurring colors. These response values were used to calculate mapping coefficients such that RGB color values could be mapped to cone color values using the following equation:

\[ Q_i = c_1R + c_2G + c_3B, \]  

where \( Q_i \) is the response value for the \( i \)th photoreceptor; \( R, G, \) and \( B \) are camera response values; and \( c_{1,3} \) are the estimated mapping coefficients. A mapping equation was calculated for each cone class. More complex models can be used but were equivalent in characterizing our dataset, thus the simplest mapping equation was used (Table S1). All linearized TIFF images were then converted from RGB response values to cone response values using a MATLAB script (Appendix S4).

MALE COLOR MEASUREMENT

Male color differences were assessed at corresponding areas on the body, anal fin, and spiny dorsal fin (Fig. 1A). After calibration, the GNU Image Manipulation Program (GIMP; version 2.8.2, available at: http://www.gimp.org/downloads/) was used to crop areas on the body and fins for analysis (Fig. 1A). These cropped portions were imported into ArcGIS.
Figure 1. (A) Crop (dotted black lines) and sampling areas (solid black lines) for color measurements on the body, anal fin, and dorsal fin. (B) Landmarks used in GM analyses. Focal species pairs used in this study including (C) *Etheostoma etnieri*–*E. flavum*, (D) *E. raneyi*–*E. zonistium*, (E) *E. ramseyi*–*E. tallapoosae*, (F) *E. barrenense*–*E. orientale*, (G) *E. inscriptum*–*E. thalassinum*, (H) *E. baileyi*–*E. coosae*, (I) *E. cervus*–*E. pyrrhogaster*, and (J) *E. lynceum*–*E. rupestre*.

**BRIEF COMMUNICATION**

**Figure 1.** (A) Crop (dotted black lines) and sampling areas (solid black lines) for color measurements on the body, anal fin, and dorsal fin. (B) Landmarks used in GM analyses. Focal species pairs used in this study including (C) *Etheostoma etnieri*–*E. flavum*, (D) *E. raneyi*–*E. zonistium*, (E) *E. ramseyi*–*E. tallapoosae*, (F) *E. barrenense*–*E. orientale*, (G) *E. inscriptum*–*E. thalassinum*, (H) *E. baileyi*–*E. coosae*, (I) *E. cervus*–*E. pyrrhogaster*, and (J) *E. lynceum*–*E. rupestre*.

(Version 9.3.1, ESRI, Redlands, CA) and each image was spatially transformed to overlay one another. Sampling areas were identified that captured the diversity of color and pattern across specimens (Fig. 1A) and 10 random points were created in each sampling area using Hawth's Tools (version 3.27, available at: http://www.spatialgeology.com/htools/download.php). Color values were sampled at each of these random points and averaged to calculate MWS and LWS values for each sampling area in each region of interest. Ten sample points were chosen to limit the chances of resampling pixel values in each area. While increasing sampling points in the dorsal fin was not possible due to the small size of the sampling areas, increasing sampling to 40 random points in the anal fin and body did not influence our results (unpublished data). In total, 42 variables (i.e., MWS and LWS scores for 12 body areas, three anal fin areas, and six spiny dorsal fin areas) were used to describe color across 192 specimens. MWS and LWS cone responses were evaluated in two different ways: (1) independently and (2) in a color opponency framework (see Supporting Information for methods).

**BODY SHAPE MEASUREMENT**

Geometric morphometrics (GMs) were used to quantify differences in body shape between species. Digital images of 2010, 2011, and 2012 specimens (n = 434) were used to digitize 10 landmarks using tpsDig2 (version 2.16, available at: http://life.bio.sunysb.edu/morph/soft-dataacq.html). All specimens used were males. Landmarks included the most anterior point on the head, the anterior junctions of the spiny and soft dorsal fins with the dorsal midline, the posterior junctions of the spiny and soft dorsal fins with the dorsal midline, the junctions of the caudal fin with the dorsal and ventral midline, the anterior junction of the anal fin with the ventral midline, the point on the ventral midline between the anterior junctions of the pelvic fins with the body, and the dorsal junction of the left pectoral fin with the body (Fig. 1B). These landmarks are similar to those used in previous studies of darter body shape (Guill et al. 2003a,b). Digitized landmarks were imported into PAleontological STatistics software (PAST, version 2.15, Hammer et al. 2001, available at: http://nhm2.uio.no/norlex/past/download.html).

Preliminary data analysis using relative warps (RWs) indicated that specimens exhibited body arching, a result of slight body posture differences during preservation, which can confound biologically relevant differences in body shape. These differences were removed using the Burnaby method described by Valentin et al. (2008) as implemented in PAST. In total, 20 variables (i.e., X and Y coordinates for 10 landmarks) were used to describe body shape in 434 specimens.


SPECIES PAIR SELECTION

Eight species pairs were chosen to represent recent divergence in darters (Fig. 1C). Focal species are members of three closely related darter subgenera, *Nanostoma, Etheostoma* s.s., and *Ulotrunga* (see Near et al. 2011 for alternative taxonomy). Species pairs were chosen to span varying levels of genetic distance within these groups; species pair selection therefore was not random, but we made no deliberate effort to select pairs that were most obviously divergent in nuptial color or body shape. Focal species in each pair do not co-occur, as closely related darter species are nearly always allopatric, consistent with the observation that allopatry is the most common geographic basis of speciation (Coyne and Orr 2004). Generally, focal species do not co-occur with other focal species, with three exceptions. *Etheostoma raneyi* and *E. lynceum* were collected from the same locality, and *E. coosae* was collected from a locality that also contains *E. rupestre* in very low frequencies (M. D. Martin, pers. obs.). The remaining 13 focal species were collected from sites that did not contain other focal species.

Species pairs also were selected to be phylogenetically independent. A species pair is phylogenetically independent from other species pairs if they do not share any branches in a tree (Mendelson 2003). In other words, evolutionary changes between a pair of species are independent from changes in another species pair, provided those changes accrue within different evolutionary lineages (Felsenstein 1985). Phylogenetically independent species pairs were chosen based on an amplified fragment length polymorphism (AFLP) phylogeny (Mendelson and Wong 2010). Two pairs are not phylogenetically independent in an alternative phylogeny (Near et al. 2011); however, by using alternative pairings phylogenetic independence can be maintained and results are similar (see Supplemental Information; Table S2). The phylogenetically independent species pairs allowed us to apply traditional statistical methods based on independent datapoints.

DATA ANALYSIS

The high dimensionality of our dataset (i.e., 42 color variables and 20 shape variables) required simplification prior to analysis. As in Arnegard et al. (2010), data dimensionality was first reduced for male color using principal components analysis (PCA). Color data were reduced to the minimum number of principal components (PCs) necessary to explain 95% of the variation in the dataset, resulting in eight PCs explaining 95.03% of variation in the LWS/MWS color dataset and 14 PCs explaining 95.67% of variation in color opponency values. Instead of traditional PCA, body shape data were analyzed using a RWs analysis (RWA). RWA essentially provides a PCA specific to body shape data. RWs are more easily interpreted in terms of shape differences than PCs and represent changes in body shape from a mean shape for all specimens. Like PCs, several RWs were identified that cumulatively explain all of the variation in a dataset; we focused on nine RWs that explained 96.27% of variation in the dataset.

Following PCA and RWA, differences between species in color and shape were assessed using multivariate analysis of variance (MANOVA) in conjunction with canonical variates analysis (CVA). The first two canonical variates (CVs) were retained to calculate an index of difference (64.82% of variation in color data; 54.86% of variation in shape data). Mahalanobis *D*, a multivariate measure of effect size, was used to quantify differences between species and is particularly suited to this application (e.g., Arnegard et al. 2010). Values for Mahalanobis *D* were calculated in R (version 2.15.1, R Core Development Team 2012, available at: http://www.r-project.org/index.html) using the “pairwise.mahalanobis()” function in the HDMD package. The square root of these values was calculated to obtain Mahalanobis *D* values. Quantitative comparisons across disparate traits can be problematic as the value of a color may be “easier” to evolve than the location of a fin or the length of a jaw bone. However, PCA and RWA scales trait space according to its own variance structure, and CVA and Mahalanobis *D* account for both within and between species differences. Therefore, each measure of difference is scaled to account for differences in variance across different trait types. After calculation, Mahalanobis *D* values were compared across color and body shape using a Wilcoxon signed-rank test in R. PCA, RWA, and MANOVA/CVA were carried out in PAST. Correlation between male color differences and body shape differences were assessed using Spearman’s r̂ estimated using the R software package.

Results and Discussion

Comparison of Mahalanobis *D* values indicate that differences in male color are larger between species than differences in body shape (Fig. 2; Table 2; MWS/LWS independent analysis: *W = 33, n = 8, P = 0.039; color opponency analysis: *W = 34, n = 8, P = 0.023*). A single PC explained 68.85% of variation in the MWS/LWS color data (Fig. 2A). Factor loadings on PC1 indicate that variation in MWS and LWS color values are roughly similar. Variation across colors in the anal fin was most pronounced, followed by colors in the dorsal fin and ventral third of the body. Color differences in the middle and dorsal third of the body were minimal (Fig. S1). Similarly, much of the variation in color opponency values (45.87%) was explained by a single PC (Fig. S2), and variation appears most pronounced on the anal fin, followed by the dorsal fin and ventral third of the body (Fig. S3).

The majority of body shape variation (54.86%) was explained by two RWs, largely characterized by differences in the length and depth of the caudal peduncle and location of the mouth (Fig. 2B). Differences in male color and body shape were not correlated...
among species pairs (i.e., species pairs that were most divergent in male color were not also most divergent in body shape differences; MWS/LWS independent analysis: \( r_s(6) = 0, P = 1 \); color opponency analysis: \( r_s(6) = 0.43, P = 0.30 \)).

Greater divergence in color pattern compared to body shape across eight species pairs suggests an important role for sexual selection in promoting divergence between closely related darter species. Male nuptial coloration is a target of sexual selection in many taxa (i.e., Kodric-Brown 1985; Hill 1990; Olsson 1994). Male color signals can increase mating success either by increasing access to mates through male competition (Korzan and Fernald 2007) or by increasing mate acceptance through female choice (Safran et al. 2005). In contrast, body shape is most often associated with habitat use (Herrel et al. 2001) or trophic ecology (Rüber and Adams 2001) and is often used to assess these ecological parameters in stream fishes (e.g., Douglas and Matthews 1992; Norton 1995; Rincón et al. 2007). Because we find that sexual signals are more divergent than a well-established ecological trait, our data support the hypothesis that sexual selection has a greater influence on phenotypic divergence than natural (ecological) selection in the early stages of divergence in darters.

Nuptial color in darters, expressed almost exclusively by males and only during the breeding season, is likely subject to sexual selection. Females have clear preferences for particular male colors. *Etheostoma barrenense* females prefer a hue of male nuptial color that most closely resembles the population average.
Table 2. Genetic distance and trait differences for each focal species pair.

<table>
<thead>
<tr>
<th>Species Pair</th>
<th>Genetic Distance</th>
<th>Male Color (Independent)</th>
<th>Male Color Opponency</th>
<th>Body Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Etheostoma</em> rayseyi–<em>Etheostoma</em> tallapoosae</td>
<td>0.041</td>
<td>2.18</td>
<td>1.69</td>
<td>0.54</td>
</tr>
<tr>
<td><em>Etheostoma</em> inscriptum–<em>Etheostoma</em> thalassinum</td>
<td>0.086</td>
<td>1.89</td>
<td>0.40</td>
<td>0.81</td>
</tr>
<tr>
<td><em>Etheostoma</em> raneyi–<em>Etheostoma</em> zonistium</td>
<td>0.100</td>
<td>3.33</td>
<td>1.54</td>
<td>0.65</td>
</tr>
<tr>
<td><em>Etheostoma</em> cervus–<em>Etheostoma</em> pyrrhogaster</td>
<td>0.101</td>
<td>0.86</td>
<td>1.20</td>
<td>0.91</td>
</tr>
<tr>
<td><em>Etheostoma</em> etnieri–<em>Etheostoma</em> flavum</td>
<td>0.101</td>
<td>9.98</td>
<td>3.17</td>
<td>0.37</td>
</tr>
<tr>
<td><em>Etheostoma</em> lynceum–<em>Etheostoma</em> rupestre</td>
<td>0.186</td>
<td>2.29</td>
<td>3.76</td>
<td>2.79</td>
</tr>
<tr>
<td><em>Etheostoma</em> barrenense–<em>Etheostoma</em> orientale</td>
<td>0.195</td>
<td>3.65</td>
<td>2.59</td>
<td>1.37</td>
</tr>
<tr>
<td><em>Etheostoma</em> baileyi–<em>Etheostoma</em> coosae</td>
<td>0.203</td>
<td>6.12</td>
<td>6.23</td>
<td>4.76</td>
</tr>
</tbody>
</table>

Pairs are ordered from most closely related to most distantly related.

(Williams et al. 2013). Females of this species also prefer conspecific over heterospecific male nuptial coloration, as do females of a sympatric congener, *E. zonale* (Williams and Mendelson 2010, 2011). In addition, male aggression biases can be clearly linked to color differences in several species, with males acting more aggressively to dummyes or live males of similar color (Williams and Mendelson 2013; M. D. Martin and T. D. Mendelson, unpubl. ms.). That said, other studies of darters outside our focal group fail to show female preference based on color (Pyron 1995; Cicotto et al. 2014); nonetheless, the preponderance of evidence suggests that male nuptial color is subject to sexual selection within our focal darter group.

In our study, color differences were most pronounced on the fins. Fin displays in darters are used by males in aggressive intrasexual interactions and when initiating courtship (M. D. Martin and T. D. Mendelson, pers. obs.; Video S1). Unlike colors on the body, colors on the fins can be masked when the fins are not held erect. Greater variation in fin coloration across species is corroborated by a phylogenetic reconstruction of snubnose darter color, in which colors on the fins have low phylogenetic signal (i.e., exhibit variation unrelated to phylogenetic relationships), whereas colors on the body exhibit significant phylogenetic signal (i.e., variation is related to phylogenetic relationships; Gumm and Mendelson 2011). Colors on the fins therefore appear to be more labile than colors on the body. Indeed, females prefer specific hues of body color in *E. barrenense* that more closely resemble the population average, suggesting a role for stabilizing sexual selection on body color (Williams et al. 2013). Directional sexual selection on fin colors would predict rapid divergence in fin colors between species, but manipulative experiments are necessary to understand the nature of sexual selection shaping these traits.

Body shape is associated with microhabitat use in darters and represents the primary axis of ecological differentiation in darters (Page and Swofford 1984). Jaw morphology can also indicate ecological divergence in fishes (Wainwright and Richard 1995) but does not differ across darter species to the same extent as body metrics (Carlson and Wainwright 2010). Our results corroborate previous work that shows that body shape and jaw morphology are most distinct among subclades (e.g., subgenera) of darters rather than among closely related species (Carlson and Wainwright 2010; Guil et al. 2010). Divergence in microhabitat use therefore may have had a stronger influence on deeper divergences in darters. A broader taxonomic sample that spans distant and recent relationships would address the relative importance of sexual and natural selection at different stages across the darter radiation (see Danley and Kocher 2001).

In our study, body shape differences between species were minimal, but the differences we did find were consistent with other morphometric analyses (Page and Swofford 1984; Guill et al. 2003b; Carlson and Wainwright 2010). Specifically, we found variation between species in the length of the caudal peduncle (e.g., the tail spanning from the posterior insertion of the anal fin to the insertion of the caudal fin) and, to a lesser extent, in the location of the mouth. A shorter, deeper caudal peduncle has been associated with darter species inhabiting faster water velocity (Page and Swofford 1984; Guill et al. 2003b). We found that *E. rupestre*, *E. lynceum*, *E. thalassinum*, and *E. inscriptum*, which are fast current species, exhibit the shortest, deepest caudal peduncles (e.g., described by large positive values on RW 1). Variation in mouth position among snubnose darters likely relates to microhabitat use (Carlson and Wainwright 2010), but the variable of greatest effect is unknown (e.g., prey capture method, prey selection, etc.) Although differences were observed across species (Fig. 2B), interpreting mouth position based on RW scores was difficult due to the large influence of caudal peduncle differences.

Spatial overlap among our focal species could influence divergence patterns, as previous work in darters suggests a diversifying role for sympatric interactions in relation to ecological characters. Co-occurrence in *Percina* darters increases the rate at which measures of body size and jaw morphology change (Carlson et al. 2009). In addition, the introduction of darters to previously unoccupied river systems can cause changes in...
the native darters over short time scales in jaw morphology (Carlson 2008). Other work, however, has found little to no trait divergence across several multispecies comparisons (Knouft 2003). If ecological characters diverge as a result of spatial overlap, our metric would likely overestimate body shape divergence that we assume is occurring in allopatry. Thus, the observed pattern of a relatively more important role for sexual selection in our study is robust to the influence of spatial overlap. Relatively less is known about the potential for reinforcement and reproductive character displacement to facilitate color pattern divergence in darters. Increased mate discrimination in sympatric species pairs or populations suggests that reinforcement may occur in darters (Martin and Mendelson 2013; Zhou and Fuller 2014), but direct measures of divergence in color patterns between allopatric and sympatric populations have not yet been made.

While our data suggest that sexual selection may have a greater influence in the early stages of phenotypic divergence in darters, ecological selection is likely also contributing. The majority of species pairs exhibit greater differences in male color than in body shape, but these indices of difference are not correlated. Some species pairs are more divergent in body shape than others, and one pair in particular exhibits greater divergence in body shape than male color. This variation in degree and type of phenotypic divergence could indicate where along the continuum each species pair lies between “ecological adaptation alone” and “sexual selection alone” (Fig. 2C). For example, divergence between E. flavum and E. etnieri, which differ greatly in nuptial color and little in body shape, may be closer to “sexual selection alone,” whereas divergence between E. lynceum and E. rupestre, which exhibit the opposite pattern, may be closer to the “ecological adaptation alone” at the end of the continuum. A deeper understanding of how these phenotypic differences contribute to reproductive isolation in darters also would help distinguish which evolutionary mechanisms are most important in darter divergence and speciation.

Like many animal groups, darters are characterized by marked differences in secondary sexual traits. Despite this common pattern in nature, evidence for speciation by sexual selection is less substantial than evidence for speciation by natural selection (i.e., ecological speciation; but see Ritchie 2007; Kraaijeveld et al. 2011). This may be due, in part, to the complex and pervasive interactions between natural and sexual selection (reviewed in Maan and Seehausen 2011; Safran et al. 2013). Comparative, trait-based studies such as this represent an important step toward understanding the process of speciation in the context of interacting natural and sexual selection. By accounting for both processes, we are able to interpret our results along a continuum of selection mechanisms (Maan and Seehausen 2011; Safran et al. 2013) instead of a typical categorical framework. Using generally applicable methods and applying that continuous framework, we are able to ask not only whether sexual selection promotes speciation, but to what degree sexual selection contributes to divergence in the presence of other selective forces.

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DATA ARCHIVING
The doi for our data is 10.5061/dryad.n28rf.

LITERATURE CITEd

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BRIEF COMMUNICATION

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Appendix S1. Linearization script.
Appendix S2. Camera sensitivity function estimation script.
Appendix S3. Mapping coefficient estimation script.
Appendix S4. Image mapping script.
Table S1. Alternative mapping equation accuracy.
Table S2. P-values for statistical tests across measures of color difference and phylogenetic hypotheses.
Figure S1. Factor loading scores on the first principal component for (A) LWS and (B) MWS scores.
Figure S2. (A) Principal components analysis for color opponency values (B) and male color and body shape differences interpreted along a continuum between “sexual selection alone” (i.e., pronounced male color differences) and “ecological adaptation alone” (i.e., pronounced body shape differences).
Figure S3. Factor loading scores on the first principal component for color opponency values.
Video S1. Video link: http://youtu.be/lCf-s8Ev8xE.