Patterns of Male Breeding Color Variation Differ across Species, Populations, and Body Size in Rainbow and Orangethroat Darters

Authors: Zhou, Muchu, Johnson, Ashley M., and Fuller, Rebecca C.

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Patterns of Male Breeding Color Variation Differ across Species, Populations, and Body Size in Rainbow and Orangethroat Darters

Muchu Zhou[1], Ashley M. Johnson[1], and Rebecca C. Fuller[1]

Sexually dimorphic coloration has been widely suggested to play a role in sexual selection and speciation. Animal colors can originate from several different biochemical pathways, which may underlie different patterns of selection and diversification. Darters of the speciose genus *Etheostoma* exhibit substantial diversity in male breeding coloration. We used digital photography and image software to comprehensively quantify male coloration in the Rainbow Darter (*Etheostoma caeruleum*) and the Orangethroat Darter (*E. spectabile*). Color traits differed across species, populations, and body sizes, with size differences contributing the most to individual color variation. The bluish colors were overall more strongly correlated with size than the reddish colors. Conversely, the reddish colors tended to be less correlated with size and better indicators of species and population identity. Finally, we determined that the bluish colored tissue contained a chromoprotein pigment, and that the reddish colored tissue contained a carotenoid pigment. The patterns of conservation and diversification in darter male coloration provide a guide for future investigations into their functional and evolutionary significance.

MULTIPLE evolutionary forces are known to act upon animal breeding coloration, including genetic drift (Wright, 1931; Wlasiuk et al., 2003; Lehtonen et al., 2009), environmental differences (e.g., sensory drive, Endler, 1991; Scott, 2001; Fuller, 2002; Maan et al., 2006), divergent sexual selection (West-Eberhard, 1983; Seehausen and Van Alphen, 1999; Seehausen and Schluter, 2004), and reinforcement (Butlin, 1989; Alatalo et al., 1994; Albert et al., 2007). These factors, particularly sexual selection, may in turn have broader implications for speciation and species richness (Carson, 1978; Dominey, 1984; Barraclough et al., 1995; Owens et al., 1999). The function and evolution of breeding coloration may also be influenced by their underlying biochemical bases, e.g., structural versus pigmentary colors (Burns et al., 2004). Carotenoid pigments are a well-studied example; being diet-limited, they have often been suggested to represent ''honest'' indicators of male quality (Olson and Owens, 1998; Griggio et al., 2007). On a macroevolutionary scale, carotenoids appear to have constrained the diversification of plumage coloration in some bird groups, though this pattern is not universal (Hofmann et al., 2006; Kiere et al., 2009; Prager and Andersson, 2010). Comparative studies of coloration across and within related species can be a useful first step in identifying traits of interest, as well as providing clues as to the factors that may have acted upon them.

Etheostoma (Teleostei: Percidae), one of the genera of fishes commonly known as darters, contains some 140 recognized species and is thus the largest freshwater fish genus in North America (Page and Burr, 1991). Species of *Etheostoma* are sexually dichromatic: the males are distinctively colored and patterned, particularly during the mating season, whereas the females are relatively drab and cryptic. There is enormous interspecific diversity in male coloration within the genus; considerable geographic color and pattern variation have also been documented within a number of species (Kuehne and Barbour, 1983; Page, 1983). Such distinctions in coloration have formed an important basis for descriptions of new species (Ceas and Page, 1997).

Male coloration in *Etheostoma* has conventionally been thought to be the product of sexual selection (Reeves, 1907; Mendelson, 2003). However, the particular mechanisms underlying sexual dichromatism and color diversity within the clade are incompletely understood. Williams and Mendelson (2010, 2011) found a female preference for conspecific male colors and patterns over heterospecifics in *E. barrenense* and *E. zonale*, suggesting that species recognition has played a role in the evolution of darter breeding coloration. However, at the intraspecific level Pyron (1995) and Fuller (2003) found female preferences for male coloration in *E. spectabile* and *E. caeruleum* to be either non-existent or unimportant to reproductive success. The complexity of male breeding coloration in most species of *Etheostoma*, consisting of a combination of bars, spots, and/or other elements (Kuehne and Barbour, 1983; Page, 1983), presents a challenge to unraveling its significance as the various components may differ in function and evolutionary history. Complex coloration that encodes multiple messages have been documented from other taxa; for example, among mammals in Carnivora, markings on the body, around the eyes, and on the tail seem to serve cryptic, physiological, and signaling purposes, respectively (Ortolani, 1999).

The Rainbow Darter (*E. caeruleum*) and the Orangethroat Darter (*E. spectabile*) are two common species with wide, partially overlapping distributions in the eastern United States. Both species belong to the subgenus *Oligocephalus* and are well suited for the study of interspecific color divergence because they are otherwise similar morphologically, ecologically, and behaviorally: both are small, benthic fish that inhabit the riffles of shallow, fast-moving streams, and have essentially the same mating season and breeding system (Winn, 1958). Male *E. caeruleum* and *E. spectabile* exhibit superficially similar blue-green and orange-red breeding colors on the head, body, and fins. The most obvious color difference between the two is the presence of red on the anal fin of male *E. caeruleum*, which is absent in male *E. spectabile* (Kuehne and Barbour, 1983; Page, 1983).

Our study aims to (1) determine how male color traits vary between and within species, (2) examine the relationship between male coloration and body size, and (3) synthesize patterns of male color variation across multiple traits and in

[1] School of Integrative Biology, University of Illinois, 606 E. Healey St., Champaign, Illinois 61820; E-mail: (MZ) zho1@illinois.edu; (AMJ) ajohns66@uiuc.edu; and (RCF) fuller@life.illinois.edu. Send reprint requests to MZ.
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Table 1. Field collection sites.

Site	Drainage	*n*		Latitude	Longitude
		E. caeruleum	*E. spectabile*		
1. Black Slough	Embarras	0	12	039°58′40″N	088°10′30″W
2. Deer Creek	Embarras	8	11	039°41′40″N	088°08′50″W
3. Hackett Branch	Embarras	8	11	039°55′20″N	088°15′40″W
4. Farr Creek	Kankakee	8	0	041°09′50″N	087°44′30″W
5. Kaskaskia Ditch	Kaskaskia	0	14	040°08′30″N	088°20′30″W
6. Page Run	Kaskaskia	0	12	039°59′40″N	088°16′00″W
7. Clear Creek	Little Wabash	0	12	039°24′40″N	088°28′20″W
8. Green Creek	Little Wabash	0	12	039°15′30″N	088°31′40″W
9. Mackinaw River	Mackinaw	0	14	040°34′20″N	088°24′30″W
10. Mackinaw Tributary	Mackinaw	0	10	040°31′50″N	088°39′10″W
11. Big Ditch	Sangamon	0	11	040°17′20″N	088°17′10″W
12. Sangamon Tributary	Sangamon	0	9	040°15′10″N	088°25′10″W
13. Wildcat Slough	Sangamon	0	4	040°21′50″N	088°14′10″W
14. Jordan Creek	Vermillion	13	0	040°04′30″N	087°49′30″W
15. Middle Fork	Vermillion	16	0	040°14′20″N	087°47′00″W
16. Salt Fork	Vermillion	6	5	040°03′20″N	088°05′30″W
17. Upper Salt Fork	Vermillion	0	9	040°09′30″N	088°04′00″W

light of the colors' biochemical origins, so as to gain insight into the potential underlying selective forces.

MATERIALS AND METHODS

Adult male *E. caeruleum* and *E. spectabile* (subsp. *spectabile*) were collected from 17 sites in central Illinois, spread over seven river drainages (Table 1, Fig. 1). While eight of these sites occur within the sympatric range of *E. caeruleum* and *E. spectabile*, we collected both species from only three of these sites. Most of the sites were small, shallow (depth <1 m) drainage steams adjacent to farms, with riffles over fine to coarse gravel; the exceptions were Middle Fork and Jordan Creek, which were relatively larger streams in forested preserves. Collecting took place using kick-seines in April and May of 2009, during the breeding season of both species. Of the 125 *E. spectabile* collected, 20 exhibited a very small amount of yellowish coloration on the anal fin. We cannot exclude the possibility that these were hybrids, which have been recorded between *E. caeruleum* and *E. spectabile* (Martin and Richmond, 1973; McLeod et al., 1980; Bossu and Near, 2009). However, in all other respects these individuals appeared typical of *E. spectabile*, and removing them from our analyses did not qualitatively change the results. The standard length, to the nearest millimeter, was measured for each fish.

Photography.—Darter coloration was measured via digital photography with a Nikon Coolpix 8700 camera. Adult *E. caeruleum* and *E. spectabile* have a two-cone visual system (long-wavelength sensitive and medium-wavelength sensitive) and lack a short-wavelength sensitive cone (M. Zhou and E. Loew, unpubl.). Therefore, the quantification of color in a human-visible color space employed by this study can still be reasonably expected to capture a large proportion of the color variation relevant to these species.

We took photographs of the fish in the lab within a day of field collection, under standard fluorescent room lighting. Each fish was anesthetized with a 0.03% tricaine methanesulfonate (MS-222) solution. MS-222 has been used as an anesthetic agent in previous quantitative studies of fish

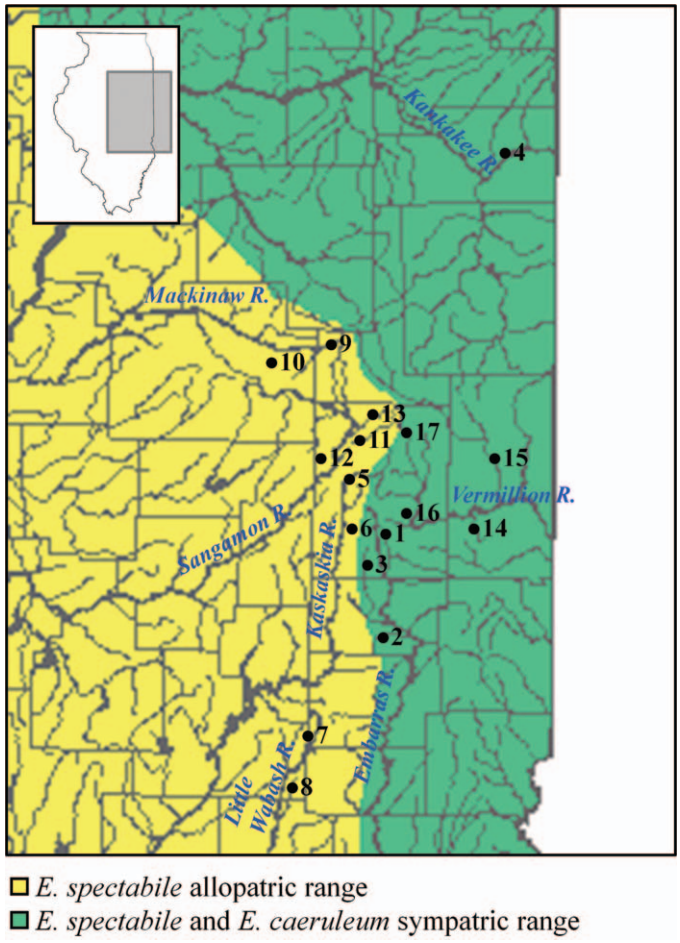

Fig. 1. Map of central Illinois showing field collection sites and geographic ranges of *E. caeruleum* and *E. spectabile.* The sites are numbered as in Table 1.

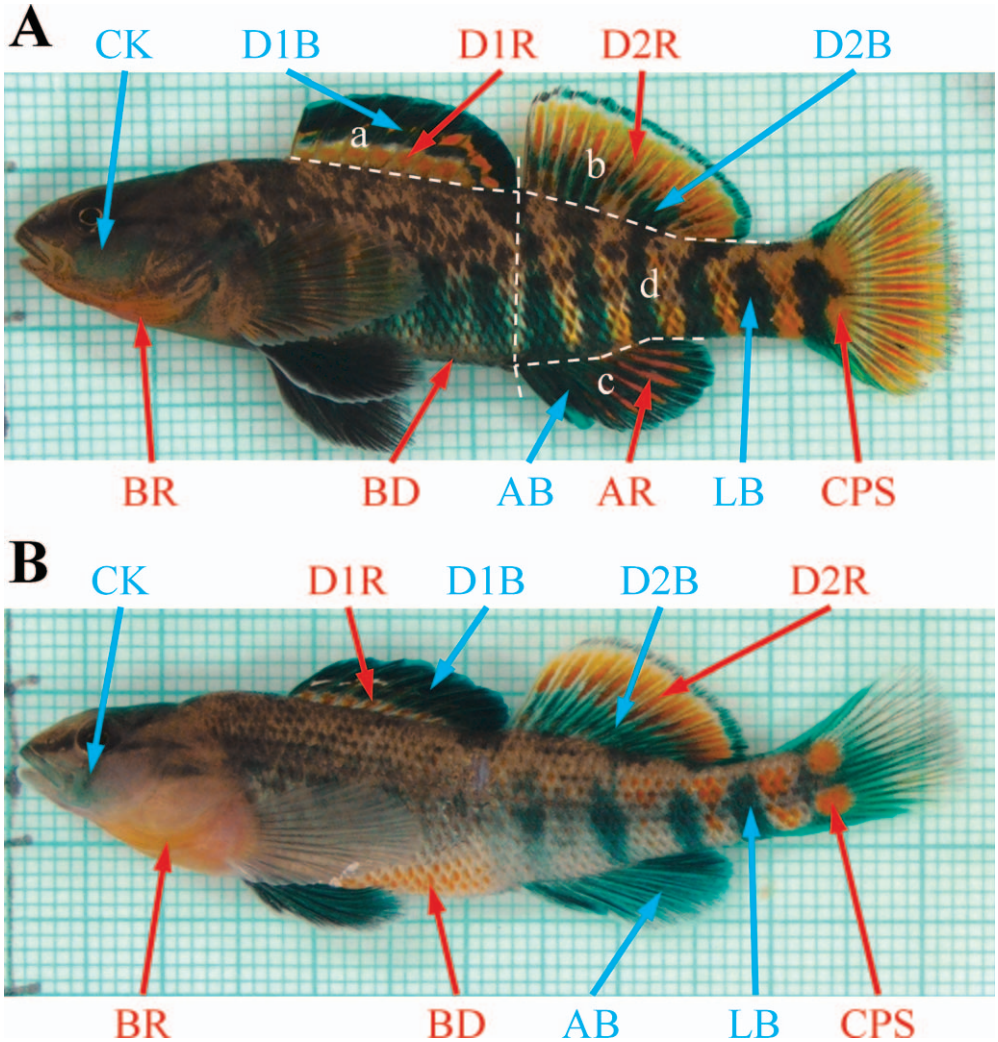

Fig. 2. Examples of (A) male *E. caeruleum* from Farr Creek, Kankakee River drainage and (B) male *E. spectabile* from Upper Salt Fork, Vermillion River drainage. Uppercase labels are abbreviations for color traits as given in Materials and Methods. Lowercase labels denote the fish regions used for blue/red area measurement: (a) first dorsal fin, (b) second dorsal fin, (c) anal fin, and (d) caudal region.

color and pattern (Endler, 1991; Yasir and Qin, 2009), and appears to maximize color expression in darters (Gumm and Mendelson, 2011). Once the fish became unresponsive to tactile stimulus, it was placed in a Petri dish filled with clean treated water to prevent the overhead lights from reflecting off the scales. The underlying background was white with a 1 mm grid. A Munsell Color X-Rite Mini ColorChecker chart (Grand Rapids, MI) was placed alongside for color standardization (details below). Five to six photographs were taken for each fish, including lateral and ventral views. When necessary, surgical probes were used to extend the fins.

Quantification of color traits.—Color standardization was performed following the method described in Bergman and Beehner (2008). Each photograph included a ColorChecker chart, consisting of 24 squares containing 18 colors and a six-step gray scale. The photographs were processed in Adobe Photoshop CS4 Extended, with the inCamera plug-in (version 4.0.1, PictoColor Software). On the ''ColorChecker'' setting, the plug-in provides a grid that we manually aligned to the squares of the ColorChecker chart. The ''check capture'' function of the plug-in was used to confirm that variation within each square was minimal (<3.0 standard deviations). The plug-in was then used to create a digital profile that adjusted the colors of the photograph according to the known values of the ColorChecker chart. Finally, the photograph was converted to the new profile.

Eleven color traits, distributed across the body and fins, were measured for each fish (Fig. 2). We attempted to measure at a spot close to the center of the color patch, avoiding obvious blemishes; since the colors on the fins are distributed across a series of patches separated by the fin rays, a patch near the middle rays was selected for measurement. If a color patch exhibited a gradation of color, a spot at the middle of the gradation was chosen. Five of the traits were categorized as ''blue'': cheek (CK), first dorsal fin blue (D1B), second dorsal fin blue (D2B), anal fin blue (AB), and lateral bar (LB; second to last from caudal fin). The remaining six traits were categorized as ''red'': branchiostegal rays (BR), first dorsal fin red (D1R), second dorsal fin red (D2R), anal fin red (AR), caudal peduncle spot (CPS; lower), and abdomen (BD). We recorded the color value of each trait in the RGB color space, which describes colors as an additive mixture of red, blue, and green (range of values 0–255). The traits were measured using the eyedropper tool in Photoshop, set to measure from a 3 × 3 pixel square. Each trait was measured three times from three separate photographs of each fish, and the RGB values averaged. We

performed repeatability analyses for each variable measured (Lessells and Boag, 1987); repeatability across the three photographs was 0.80±0.02 (S.E.).

For further analyses, we converted the RGB values a luminance channel *R+G+B* and two color channels *(R−G)/(R+G)* and *(G−B)/(G+B)* (Endler, 2012), hereafter referred to as the red-green difference (R-G) channel and the green-blue difference (G-B) channel. A positive R-G value indicates a color with a stronger red component and a negative value a color with a stronger green component; correspondingly a positive G-B value indicates a color with a stronger green component and a negative value a color with a stronger blue component.

Quantification of blue/red area.—As ''blue'' and ''red'' colors constitute the two major aspects of male coloration, we also measured the proportional area of blue and red on four regions of the fish: first dorsal fin (D1B area and D1R area), second dorsal fin (D2B area and D2R area), anal fin (AB area and AR area), and caudal region (CRB area and CRR area), with the anterior limit defined by a straight line drawn between the origins of the second dorsal and anal fins, and including the entire caudal fin (Fig. 2A). The anterior portion of the body could not be analyzed due to variable occlusion by the pectoral fin. The total area of each region was obtained by manually tracing the region with the polygonal selection tool in ImageJ (version 1.43u, Wayne Rasband), and counting the number of pixels using the histogram tool.

We obtained the areas of blue and red coloration within each region using the Threshold Colour plug-in (version 1.10, G. Landini) for ImageJ. The plug-in allows colors to be stopped above or below a set threshold in a color coordinate space. The photographs were processed in the CIE *Lab* color space; we chose to use *Lab* instead of RGB for this process because it describes colors using the coordinates L* (lightness), a* (red/green), and b* (blue/yellow), which proved more convenient for isolating blue and red in our photographs. Based on visual examination, we selected threshold values (range 0–255) that would be conservative with regard to the color area included. To isolate blue coloration, L* was set to stop colors above 200/255, and b* was set to stop colors above 130/255. To isolate red coloration, the L* filter was kept at 200/255, and a* was set to stop colors below 125/255. After the filters were applied, we transformed the image into binary black and white and counted the black pixels within each color region using the histogram tool. Finally, the pixel counts were used to calculate the proportion of blue and red within each region.

Statistical analyses.—All statistical analyses were performed in SAS (version 9.1, SAS Institute, Cary, NC). Standard length was compared between species and sites using a linear mixed model (MIXED procedure in SAS). Due to the low number of sites that contained both species, we nested sites within species and set it as a random effect. Similarly, we elected not to include drainage as an effect because only two of the seven drainages sampled yielded both species. Random effects in this and subsequent models were assessed using exact F tests.

Between- and within-species variation in luminance, color, and blue/red area were synthesized across multiple traits via principal components analyses (PCA) using correlation matrices (PRINCOMP procedure in SAS). The variables included in each PCA are given in Figure 3; the luminance and color channels of anal fin red were excluded because this trait was not discernible on most *E. spectabile*. The first two principal components of each PCA were then analyzed using linear mixed models for the effects of species and site nested within species, with standard length as a covariate and including standard length × species and standard length × site within species interactions. Site and its interaction with standard length were both treated as random effects.

To determine the aspects of male coloration that were most predictive of species identity, we performed a stepwise discriminant function analysis using the forward selection method on *E. caeruleum* and *E. spectabile* combined (STEPDISC procedure in SAS). The variables included were the luminance and color channels for ten of 11 color traits (again excluding anal fin red) and the eight color areas. Discriminant function analyses were also performed for each species alone to determine the characters that were most predictive of population identity; anal fin red was included for *E. caeruleum* but excluded for *E. spectabile*.

Pigment characterization.—We extracted pigments from male *E. caeruleum* ($n = 6$) and *E. spectabile* ($n = 5$) that were collected in May 2011 and not used in any other analyses. The fish were euthanized with an overdose of MS-222; red colored tissue was obtained from the second dorsal fin, and blue colored tissue was obtained from the first dorsal or the anal fin. The red colored tissue was ground using a mortar and pestle in 1 mL of 1% NH_4OH until there were no visible clumps of pigmented tissue. The pigment was then transferred to a 1:1 solution of hexane and tert-butyl methyl ether by vigorous vortexing. The absorbance of the solution was measured from 270–700 nm on a UNICO 2800UV/VIS spectrophotometer. Because the quantity of pigment in the blue colored tissue was extremely low, the tissue was ground in 200 μL of 1% NH_4OH, and the absorbance was measured from 220–750 nm using a NanoDrop ND-1000 spectrophotometer.

RESULTS

Body size.—The standard length was 47.6±0.78 mm (S.E.; range 33–64 mm) for *E. caeruleum* and 46.4±0.55 mm (36–64 mm) for *E. spectabile*. Standard length did not differ between species ($F_{1,18} = 1.51$, $P = 0.234$), but did vary between sites nested within species ($F_{18,187} = 4.77$, $P < 0.0001$).

Luminance.—Variation in luminance was largely driven by body size in *E. caeruleum* and *E. spectabile*. The first principal component (PC1) accounted for 42.9% of total variation, dwarfing the second principal component (PC2) which accounted for 14.9% of total variation. PC1 scores were strongly negatively correlated with standard length and did not differ between species (Table 2, Fig. 4A). As all of the traits loaded positively onto PC1 (Fig. 3A), larger males were darker in both species. PC1 scores also varied among sites, though this effect was much smaller than the effect of size (Table 2); the lightest males overall appeared to be *E. spectabile* from Big Ditch and Wildcat Slough (Sites 11 and 13 in Fig. 5A). PC2 scores differed among sites and not between species or with standard length (Table 2). The largest loadings on PC2 were on blue in the dorsal fins, first dorsal fin, and on the branchiostegal rays (Fig. 3B), implying

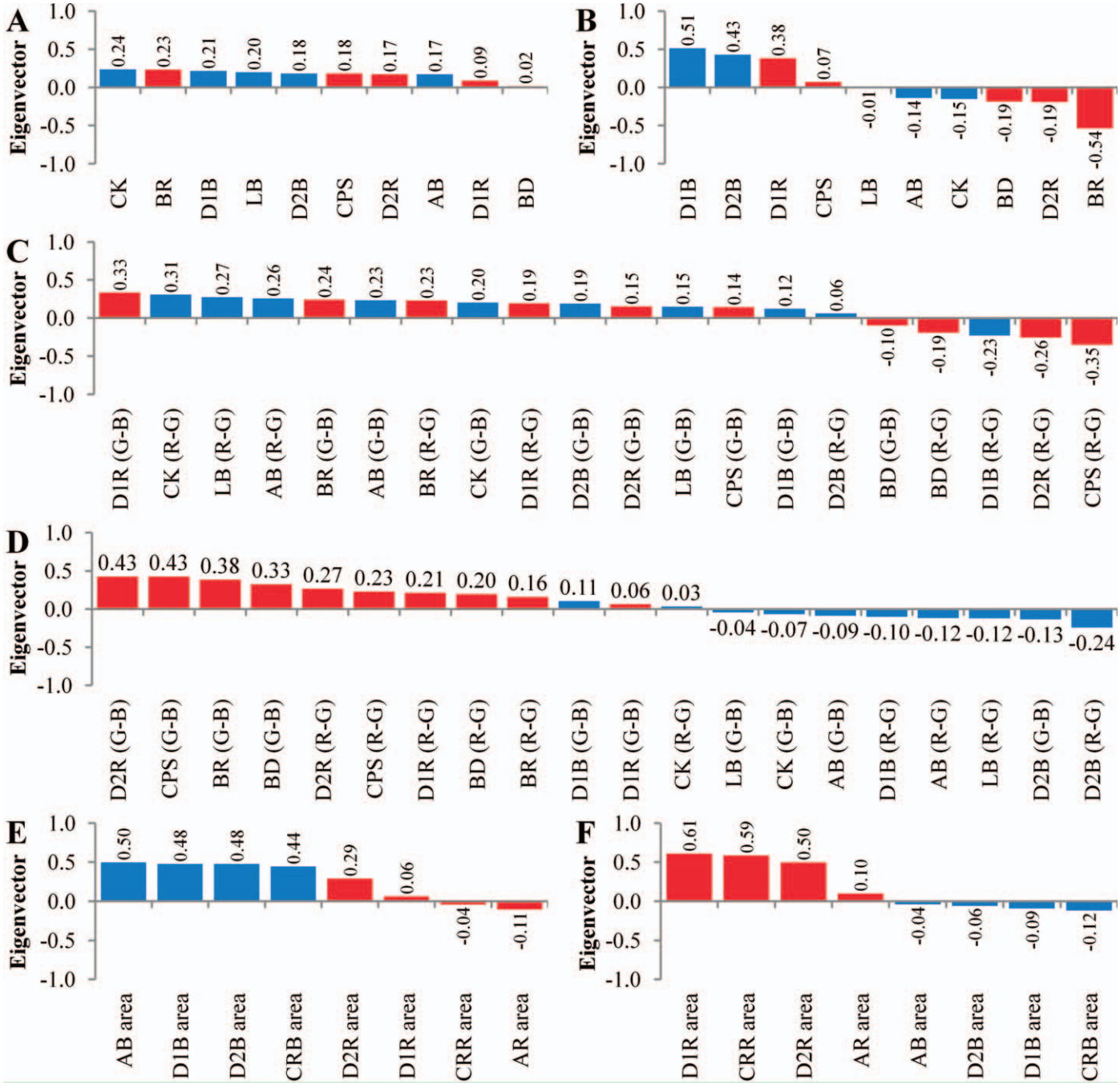

Fig. 3. Eigenvector loadings, ordered by size, on all variables included in the principal component analyses, showing (A) luminance PC1, (B) luminance PC2, (C) color PC1, (D) color PC2, (E) blue/red area PC1, and (F) blue/red area PC2. Variable abbreviations are as given in Materials and Methods.

that, for example, male *E. caeruleum* from Jordan Creek had relatively the lightest dorsal fins and darkest branchiostegal rays (Site 14 in Fig. 5A).

Color.—The first and second principal components captured relatively small amounts of the total variation in the color channels (24.4% and 15.7%, respectively), suggesting that color variation was more complex than variation in luminance or blue/red area. PC1 exemplifies this observation, representing an axis of variation affected by species, site, and standard length (Table 2). Blue traits loaded more uniformly onto PC1 than red traits, with four of the five blue traits loading positively in both R-G and the G-B channels. Of the five red traits, two loaded positively onto the R-G and G-B channels, two negatively onto both, and one negatively onto R-G and positively onto G-B (Fig. 3C). Thus, color variation in the blue traits appears to be more conservative than in the red traits.

From the trait loadings on PC1, broad trends in color variation can be deduced. PC1 scores were negatively correlated with body size (Fig. 4B), indicating that larger fish of both species tended to be bluer on the cheek, second dorsal fin, anal fin, and lateral bar (lower R-G and G-B values), and greener on the first dorsal fin (lower R-G and higher G-B values). As for the red characters, larger fish tended to be redder on the second dorsal fin and caudal

Table 2. Analyses of variance for color traits.

	Effect	DF	*F*	*P*
Luminance				
PC1	species	1, 18	0.27	0.608
	standard length	1, 18	96.96	<0.0001
	standard length × species	1, 18	1.65	0.215
	site (species)	18, 167	1.89	0.020
	standard length × site (species)	18, 167	1.51	0.091
PC2	species	1, 18	3.48	0.078
	standard length	1, 18	0.71	0.412
	standard length × species	1, 18	0.29	0.594
	site (species)	18, 167	1.69	0.045
	standard length × site (species)	18, 167	1.15	0.312
Color				
PC1	species	1, 18	14.56	0.001
	standard length	1, 18	58.23	<0.0001
	standard length × species	1, 18	6.12	0.024
	site (species)	18, 167	2.66	0.001
	standard length × site (species)	18, 167	2.25	0.004
PC2	species	1, 18	3.14	0.093
	standard length	1, 18	2.14	0.161
	standard length × species	1, 18	3.58	0.075
	site (species)	18, 167	2.60	0.001
	standard length × site (species)	18, 167	2.86	0.0002
PC3	species	1, 18	10.33	0.005
	standard length	1, 18	83.87	<0.0001
	standard length × species	1, 18	21.31	0.0002
	site (species)	18, 167	1.11	0.347
	standard length × site (species)	18, 167	0.99	0.478
Blue/red area				
PC1	species	1, 18	11.28	0.004
	standard length	1, 18	153.26	<0.0001
	standard length × species	1, 18	8.43	0.010
	site (species)	18, 167	1.31	0.187
	standard length × site (species)	18, 167	1.43	0.121
PC2	species	1, 18	1.13	0.302
	standard length	1, 18	0.00	0.968
	standard length × species	1, 18	1.65	0.215
	site (species)	18, 167	13.09	<0.0001
	standard length × site (species)	18, 167	2.83	0.0003

peduncle spot (higher R-G and lower G-B values), more orange on the abdomen (higher R-G and G-B values), and less orange on the first dorsal fin and branchiostegal rays (lower R-G and G-B values). Similarly, most *E. spectabile* had lower PC1 scores than *E. caeruleum* (Fig. 4B), indicating that the above color trends in larger versus smaller fish was also applicable to *E. spectabile* versus *E. caeruleum*. PC1 scores further varied among populations; for example, the bluest males seemed to be *E. caeruleum* from Jordan Creek (Site 14 in Fig. 5B). Finally, the relationship between size and PC1 scores varied between species and among populations (Table 2).

PC2 scores differed among populations, with a population by standard length interaction though no standard length correlation overall (Table 2). Almost all the red traits loaded positively and almost all the blue traits loaded negatively onto PC2 (Fig. 3D), suggesting a consistent intraspecific axis of color variation in which the males of some populations were overall bluer (lower R-G and G-B values) in their blue traits and more orange (higher R-G and G-B values) in their red traits than males elsewhere. For example, male *E. spectabile* from Kaskaskia Ditch appeared to have the bluest/most orange coloration (Site 5 in Fig. 5B).

Blue/red area.—The pattern of variation in the proportional areas of blue starkly differed from that in the proportional areas of red. PC1 accounted for 41.0% of total variation and primarily reflected the differences in blue area, as all four blue areas had large positive loadings while three of the four red areas had loadings close to zero (Fig. 3E). The relative amount of blue coloration increased with body size in both species: *E. spectabile* had more blue area than *E. caeruleum* but showed a weaker correlation with size (Fig. 4C). Of the red areas, only the amount of red on the second dorsal fin increased with body size and differed between species. Linear regression analyses of each of these areas against standard length corroborated the strong correlations between blue areas and size, and conversely the lack of

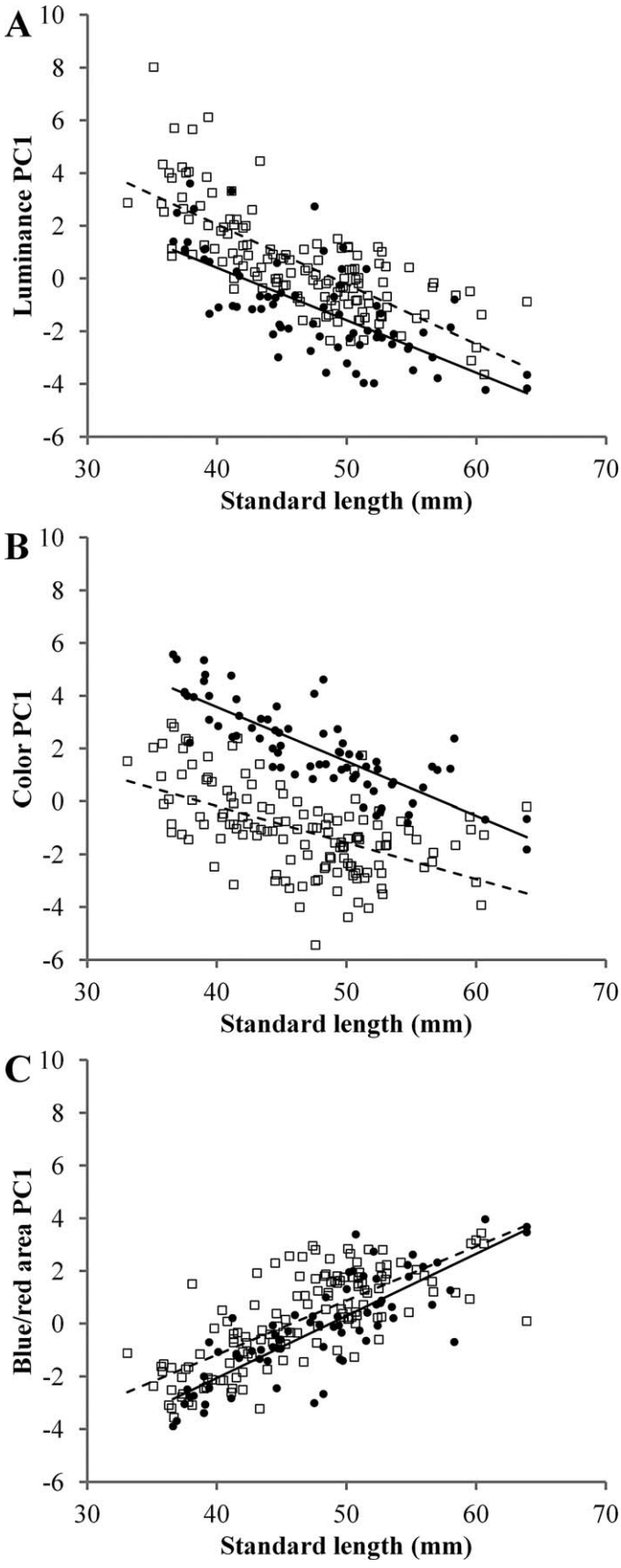

Fig. 4. PC1 scores versus standard length for all individuals, showing (A) luminance, (B) color, and (C) blue/red area. Individuals of *E. caeruleum* are represented by closed circles and the solid line, individuals of *E. spectabile* by open squares and the dashed line.

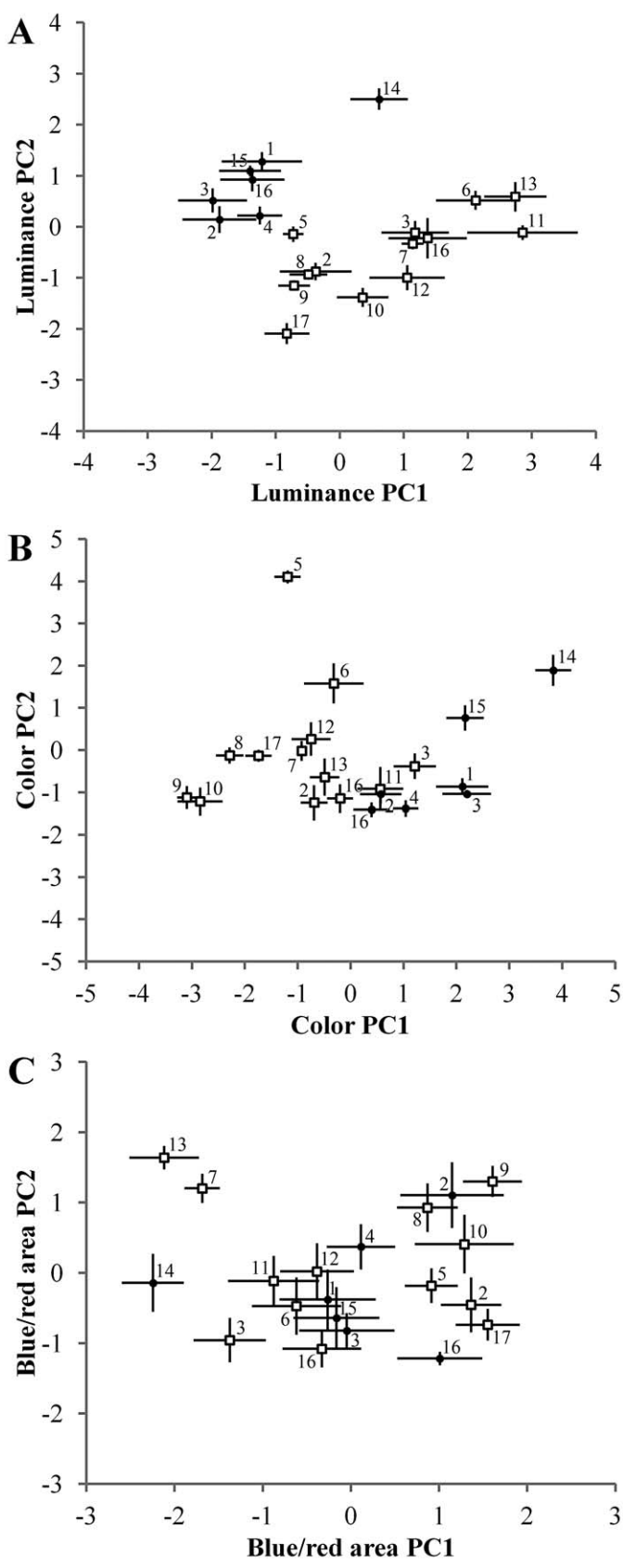

Fig. 5. Mean PC1 versus PC2 scores (± S.E.) for all populations, showing (A) luminance, (B) color, and (C) blue/red area. Populations of *E. caeruleum* are represented by closed circles and populations of *E. spectabile* by open squares. The sites are numbered as in Table 1.

Table 3. Standard length correlations for blue/red areas.

Trait	β	DF	*F*	*P*
Blue areas				
D1B area	0.017	1, 205	162.5	<0.0001
D2B area	0.011	1, 205	101.0	<0.0001
AB area	0.026	1, 205	242.6	<0.0001
CRB area	0.013	1, 205	137.8	<0.0001
Red areas				
D1R area	0.001	1, 205	1.91	0.168
D2R area	0.011	1, 205	61.0	<0.0001
AR area	0.0002	1, 205	0.07	0.796
CRR area	0.001	1, 205	1.91	0.216

correlations between red areas and size, except on the second dorsal fin (Table 3).

As opposed to PC1, PC2 primarily captured differences in red area: three of the four red areas (the exception being the anal fin) had large positive loadings while all four blue areas had loadings close to zero (Fig. 3F). PC2 accounted for 23.5% of total variation and differed among sites, with a site by standard length interaction (Table 2). Different populations would therefore appear to vary in the amount of red coloration; for example, male *E. spectabile* from Wildcat Slough had the largest areas of red coloration on their bodies and dorsal fins (Site 13 in Fig. 5C).

Discriminating between species and populations.—The between-species discriminant function analysis yielded a model with 20 predictor variables, of which ten represented blue traits and ten red. The model accounted for 92.6% of interspecific variation, indicating that it could distinguish between species with high accuracy. The within-species discriminant function models were less successful: the model for *E. caeruleum* alone accounted for 62.4% of intraspecific variation and the model for *E. spectabile* alone accounted for 69.1% of intraspecific variation. The *E. caeruleum* model included 18 predictors, nine blue and nine red, while the *E. spectabile* model included eight predictor variables, three blue and five red (Table 4).

The best predictor in all three models was a variable associated with a red trait: anal fin red area for the interspecific model and different aspects of second dorsal fin red for the intraspecific models of *E. caeruleum* and *E. spectabile* (Table 4). Anal fin red area was by far the best predictor of species identity, accounting for 63.2% of interspecific variation. No single variable or suite of variables was highly predictive of both species and population identity. Four variables occurred in both the between- and within-species discriminant function models, of which three were from red traits: first dorsal fin red (G-B channel), second dorsal fin blue (R-G channel), and second dorsal fin red (luminance and R-G channel).

Pigment characterization.—Different pigments were extracted from the blue and red colored tissues. From the blue colored tissue of both species, we obtained pigment that exhibited a decrease in absorbance centered on the blue-green region (500–530 nm). The profile of the absorption spectra, with rises in absorbance between 270–300 nm, 370–400 nm, and 670–690 nm (Fig. 6A), was consistent with that of a blue chromoprotein pigment previously described from *E. caeruleum* (Boone, 2011). The pigment from the red colored tissue in both species exhibited absorption spectrum profiles characteristic of a carotenoid, with absorption maxima at ~445 nm and ~470 nm (Fig. 6B).

Table 4. Discriminant function analyses.

Both species		*E. caeruleum*		*E. spectabile*	
Trait	ASCC	Trait	ASCC	Trait	ASCC
AR area	0.632	D2R G-B	0.112	D2R lum*	0.120
BR lum	0.738	D2B lum	0.176	CK R-G	0.226
AB lum	0.843	BD G-B	0.220	D1R G-B*	0.316
CK G-B	0.865	BD lum	0.282	CPS G-B	0.402
BD R-G	0.873	CRB area	0.322	D1R area	0.464
BD lum	0.880	D2R R-G*	0.383	D2B R-G*	0.572
AB area	0.890	D2B G-B	0.425	D2B area	0.617
BR R-G	0.900	D1R area	0.455	D2R R-G*	0.691
AB R-G	0.903	D2B R-G*	0.480		
D2B R-G*	0.908	D1B G-B	0.507		
D2R lum*	0.910	D1R G-B*	0.523		
CPS lum	0.913	CRB area	0.542		
D1R G-B*	0.915	CK R-G	0.561		
D2R R-G*	0.917	AB lum	0.578		
LB G-B	0.919	D1B area	0.593		
LB lum	0.922	D1B lum	0.598		
AB B-B	0.922	D2R lum*	0.610		
D1R lum	0.923	CPS G-B	0.624		
D1B area	0.924				
CPS R-G	0.925				
CRB area	0.926				

* common to between- and within-species models

DISCUSSION

Between-species variation.—Male breeding coloration is an important diagnostic character in the darter genus *Etheostoma*, whose species often exhibit strong sexual dichromatism (Ceas and Page, 1997; Ceas and Burr, 2002; Powers et al., 2003). We found that male *E. caeruleum* and *E. spectabile* differed across multiple aspects of their coloration. The proportional area of red coloration in the anal fin was the best trait for differentiating these species; this result is consistent with previous qualitative descriptions that reported the presence of red on the anal fin in *E. caeruleum* but not in *E. spectabile* as the main color difference between them (Kuehne and Barbour, 1983; Page, 1983). Anal fin red area was also highly conserved; unlike the other blue/red areas measured, it was neither correlated with standard length nor varied substantially among populations. If the red on the anal fin plays a role in cross-species signaling, it may have been subject to stabilizing selection.

The diversity of male breeding coloration among darters may play a role in behavioral isolation, which is an important species reproductive barrier in this group (Mendelson, 2003). Winn (1958) observed that male *E. caeruleum* and *E. spectabile* did not act aggressively toward males of the other species. Since both *E. caeruleum* and *E. spectabile* compete with conspecific rivals for spawning opportunities (Winn, 1958; M. Zhou and R. Fuller, unpubl.), inappropriate heterospecific aggression may be costly in terms of lost time and effort. In the sympatric Splendid Darter (*E. barrenense*)

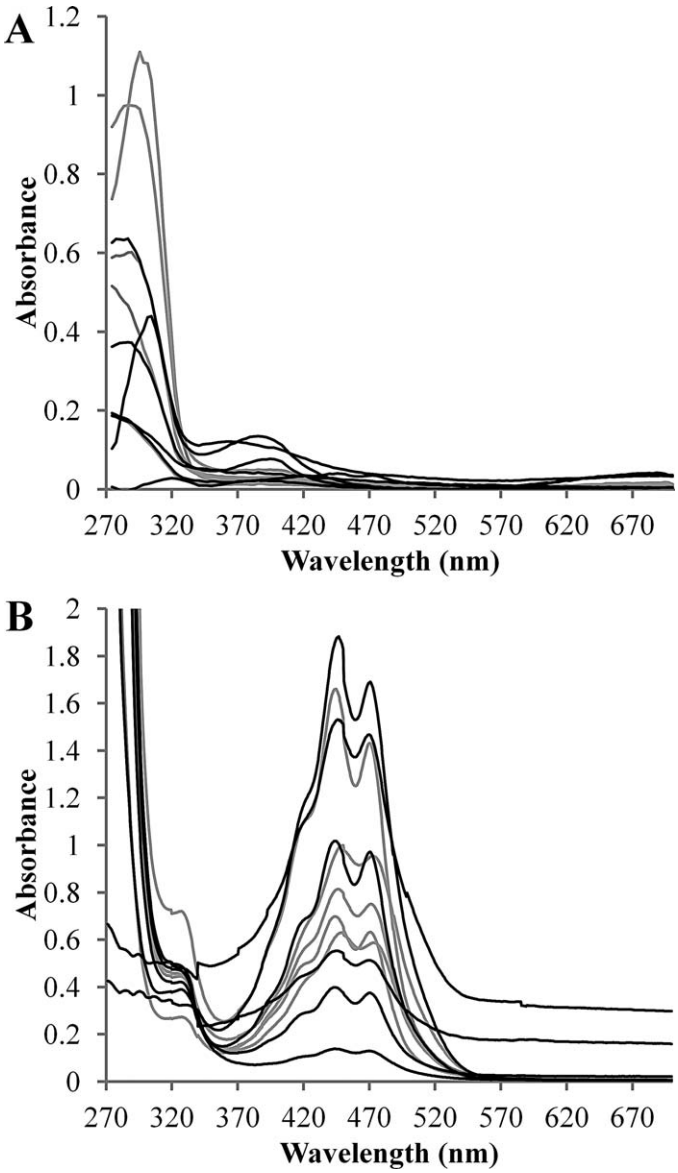

Fig. 6. Absorption spectra for (A) pigment obtained from blue colored tissue and (B) pigment obtained from red colored tissue. Individuals of *E. caeruleum* are represented by black lines, individuals of *E. spectabile* by gray lines.

and Banded Darter (*E. zonale*), male coloration appears to mediate species discrimination during both female–male and male–male interactions (Williams and Mendelson, 2010, 2011). The range of interspecific color differences we found suggests that behavioral isolation between *E. caeruleum* and *E. spectabile* may similarly be based on male coloration and on anal fin red coloration in particular.

Within-species variation.—Multiple population-level differences in male breeding coloration were present, though these effects were invariably smaller than the effects of species or standard length. Interestingly, our principal component analyses revealed patterns of variation in luminance, color, and blue/red area that solely represented population differences, independent of species or overall body size effects (the second principal components). At least for color, we found an intraspecific axis in which almost all the red traits varied in the same direction and almost all the blue traits varied in the opposite direction, suggesting a genetic or plastic response to a factor that acts on red and blue coloration as a whole rather than on particular parts of the fish.

Several non-exclusive forces may explain one or more of these among-population differences. One possibility is that male coloration has diverged under non-selective processes (e.g., genetic drift); since *E. caeruleum* and *E. spectabile* inhabit small streams near the headwaters of river drainages and are not known to be highly mobile (Winn, 1958), populations in different streams may be sufficiently isolated for genetic differentiation. Another possibility is that environmental differences (e.g., lighting, predation, competition) may have selected for different colors in different streams. In particular, overall variation in the carotenoid-based red traits may reflect differences in the available food supply at different locations, as animals must obtain carotenoids from their diet (Olson and Owens, 1998). Further study is needed to examine these possibilities.

Size-based variation.—Body size was closely associated with the blue components of male breeding coloration, and to a lesser extent with the red components. Luminance was consistently correlated with size, with larger males darker overall than smaller males. The effect of size on color was largely consistent for blue traits, which were bluer/greener in larger males, but not for red traits, which could be more or less orange/red in larger males depending on the trait. Similarly, blue area was strongly correlated with size on all four fish regions, while the same was true of red area only on the second dorsal fin.

The correlation between male coloration and body size may suggest that the former—and blue coloration in particular—advertises the latter. Male size is positively correlated with reproductive success in a number of fish species (Thompson, 1986; Hastings, 1988; Magnhagen and Kvarnemo, 1989; Maekawa et al., 1994; Jacob et al., 2009; Serbezov et al., 2010). Anecdotal observations of both *E. caeruleum* and *E. spectabile* have reported that sexually mature yearling males, which are less colorful than older males, are minimally successful in spawning with females and elicit less aggression from older males (Reeves, 1907; Winn, 1958). Experiments have also shown that larger males are better able to monopolize spawnings with females, and can thus presumably fertilize a higher proportion of her eggs (Fuller, 1999; M. Zhou and R. Fuller, unpubl.). Therefore, blue coloration in these species may signal male quality, either in terms of competitive ability in male–male interactions or attractiveness in male–female interactions. The expression of male ornaments is correlated with body size and/or other metrics of reproductive quality in many taxa (Kodric-Brown and Brown, 1984; Alatalo et al., 1988). For example, in male Blue Grosbeaks (*Guiraca caerulea*), both blueness and the amount of blue coloration is positively correlated with body size, territory size, and feeding of nestlings (Keyser and Hill, 2000). Similarly, the blueness of rump feathers in male Blue-black Grassquits (*Volatinia jacarina*) is positively correlated with body size and may play a role in male–male displays (Doucet, 2002). As there is little evidence that female preference for more or less colorful males is important for reproductive success in either *E. caeruleum* or *E. spectabile* (Pyron, 1995; Fuller, 2003), if there are component(s) of male coloration that advertise size in these species, they may be involved in male–male aggression.

Overall patterns and conclusion.—Several overarching patterns became evident when examining male breeding color variation in *E. caeruleum* and *E. spectabile*. First, body size appeared to be a larger source of variation than either species or population differences, as evidenced by the predominant effect of standard length on the first principal components of luminance, color, and blue/red area. Population was invariably the smallest contributor to total variation, and moreover included axes of variation that were decoupled from species- and size-based effects.

The coloration on the first and second dorsal fins seemed to be particularly diverse. The best predictors of population identity within *E. caeruleum* and *E. spectabile* were both from traits located on the second dorsal fin. Additionally, the four predictor variables shared by the between- and within-species discriminant function models were all from traits located on the dorsal fins. During the breeding season, male *E. caeruleum* and *E. spectabile* perform dorsal fin flaring displays toward both conspecific rivals and males of the other species (M. Zhou and R. Fuller, unpubl.). If the dorsal fins play an important signaling role in these species, sexual selection may have driven color divergence on these parts more than elsewhere on the fish.

Finally, we observed broadly differing patterns for the blue versus the red components of male breeding coloration. Variation across the blue traits was more consistent and more strongly related to body size, while variation across the red traits tended to be more diverse and more associated with interspecific and intraspecific differences. The different pigments responsible for blue versus red coloration in *E. caeruleum* and *E. spectabile* may underlie these different patterns. While the evolutionary significance of the novel blue chromoprotein pigment is virtually unknown (Boone, 2011), numerous studies have examined the possible condition-dependent nature of carotenoid-based male ornaments (Olson and Owens, 1998; Møller et al., 2000; Cotton et al., 2004; Griffith et al., 2006).

The apparent lability of carotenoid-based red coloration across and within *E. caeruleum* and *E. spectabile*, whether environmentally or genetically based, also contrasts with the view that carotenoid signals are evolutionarily constrained by their utility as ''honest'' signals (Prager and Andersson, 2010). There may not be a single rule governing the conservation or diversification of carotenoid signals across animal taxa; for example, Hofmann et al. (2006) found continuous variation and multiple character shifts in plumage over a yellow-red range within the cacique lineage (*Cacicus*, *Clypicterus,* and *Ocyalus*), implying high lability for these carotenoid-based colors. On the other hand, Kiere et al. (2009) found that plumage variation is not continuous but rather falls into discrete yellow and red categories (suggesting constraint) in the New World orioles (*Icterus*), which are closely related to caciques. Our results suggest that at least in *E. caeruleum* and *E. spectabile*, carotenoid-based red coloration may be more evolutionarily labile than blue coloration. In the *Etheostoma* subgenus *Ulocentra*, Gumm and Mendelson (2011) found a high degree of lability in all color classes (yellow, orange, red, and blue/green); thus, the macroevolutionary patterns of color diversity may vary across darter lineages as well.

Multiple factors appear to influence male breeding coloration in *E. caeruleum* and *E. spectabile*, affecting various components of the coloration in different ways. The blue components of the color pattern tend to vary along similar axes and are more strongly associated with body size, suggesting that they may play a role in reproductive interactions. The red components of the color pattern tend to vary along more diverse axes across and within species, suggesting they may play a role in interspecific or intraspecific discrimination. The coloration of the dorsal and anal fins may be of particular importance in signaling. These results offer promising avenues for further research into darter color evolution and diversification.

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