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The evolution of multi-component visual signals in darters (genus *Etheostoma*)

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Abstract   As complex traits evolve, each component of the trait may be under different selection pressures and could respond independently to distinct evolutionary forces. We used comparative methods to examine patterns of evolution in multiple components of a complex courtship signal in darters, specifically addressing the question of how nuptial coloration evolves across different areas of the body. Using spectral reflectance, we defined 4 broad color classes present on the body and fins of 17 species of freshwater fishes (genus *Etheostoma*) and quantified differences in hue within each color class. Ancestral state reconstruction suggests that most color traits were expressed in the most recent common ancestor of sampled species and that differences among species are mostly due to losses in coloration. The evolutionary lability of coloration varied across body regions; we found significant phylogenetic signal for orange color on the body but not for most colors on fins. Finally, patterns of color evolution and hue of the colors were correlated among the two dorsal fins and between the anterior dorsal and anal fins, but not between any of the fins and the body. The observed patterns support the hypothesis that different components of complex signals may be subject to distinct evolutionary pressures, and suggests that the combination of behavioral displays and morphology in communication may have a strong influence on patterns of signal evolution [*Current Zoology* 57 (2): 125–139, 2011].

Keywords   Comparative method, *Etheostoma*, Independent evolution, Nuptial coloration

Sexual selection commonly results in signaling traits that are exaggerated and complex, consisting of multiple morphological and behavioral features (Darwin, 1871). A number of non-mutually exclusive hypotheses have been proposed to explain the function of complex signals (reviewed in Candolin, 2003; Hebets and Papaj, 2005), and these hypotheses can be used to generate evolutionary predictions (Hebets and Papaj, 2005). For example, the multiple message hypothesis proposes that each component of a complex signal may indicate a different aspect of male quality (e.g., Moller and Pomiankowski, 1993; Johnstone, 1997). If each component thus represents a unique quantity of information, then the different components of a multicomponent signal may not be predicted to covary or coevolve (Candolin, 2003; Hebets and Papaj, 2005). In contrast, the redundant signal hypothesis proposes that signal components are redundant and increase accuracy of the information conveyed (e.g., back-up cues, Moller and Pomiankowski, 1993; Johnstone, 1997). Under this hypothesis, each signal contains the same information, such that signal components should covary with each other and with the quality of the signaler (Hebets and Papaj, 2005). Other hypotheses of complex signal func-

tion predict a negative covariance among the components of a multicomponent signal. For example, a 'multitasking' hypothesis (sensu Hebets and Papaj, 2005) proposes that components of a signal may constrain each other, resulting in a signal production trade off (e.g. Podos, 1997) and a tight negative covariance among signal components. Thus, evolutionary patterns of covariance among signal components can provide a signature of varying selection pressures and underlying functional hypotheses.

Empirical examples show variation in signal components may coevolve in response to multiple selection pressures. When conspicuous ornaments combine a morphological ornament with a behavioral display element, selection for morphology (e.g., color) and display are typically related to maximize expression of the traits (Peek, 1972; Prum, 1990, McLennan, 1991, 1996; Endler, 1992; Rosenthal et al., 1996, reviewed in Rosenthal, 2007). For example, in fishes, behavioral displays and morphological color traits may enhance each other (*Poecilia reticulata*, Kodric-Brown and Nicoletto, 2001; *Gasterosteus aculeatus*, McLennan and McPhail, 1990). Alternatively, evolution of the morphological trait and display behavior may be uncoupled

(e.g., Clark and Utez, 1990, 1992; Itzkowitz and Iovine, 2007; Suk and Choe, 2008). A recent study of *Sceloporus* lizards found that blue coloration and behavioral displays are decoupled, as some species have lost the coloration yet retain the behavioral display (Quinn and Hews, 2010). Further, a comparative phylogenetic study of 59 lizard species found no significant correlations between coloration and display behavior (Weins, 2000).

Phylogenetic comparative methods are especially appropriate for elucidating patterns of co-evolution among different components of a multicomponent signal. Comparative studies of complex color signals in particular commonly use ancestral state reconstruction to infer directions of evolutionary change and patterns of trait gains and losses (reviewed in Omland and Hoffmann, 2006). Additionally, causation in these studies is inferred through correlations between changes in coloration and ecological variables (Endler and Thery, 1996; Weins, 1999; McNaught and Owens, 2002; Gomez and Thery, 2004; Ord and Martins, 2006; Cummings, 2007; Doucet et al., 2007; Stuart-Fox et al., 2007). Lacking, however, are studies that have evaluated correlated changes across phylogenies in individual components of multicomponent color signals (but see Kemp et al., 2005).

Darters of the genus *Etheostoma* are particularly suited for testing hypotheses about the evolution of multicomponent color signals as males express multiple colors across multiple body regions. *Etheostoma* is the largest genus of North American freshwater fishes and likely diverged from other darter genera over 20 mya (Near and Keck, 2005). The subgenus *Ulocentra* (Bailey and Etnier, 1988), commonly known as snubnose darters, contains approximately 19 species, excluding very recent and ongoing taxonomic revisions elevating some populations to distinct species (Powers and Mayden, 2007). *Ulocentra* was shown to be a monophyletic subgenus by Porter et al. (2002), and a recent study using amplified fragment length polymorphisms (AFLPs) corroborated that result and provided a robust estimate of species relationships within the group (Mendelson and Wong, 2010). Snubnoses appear to be relatively old, for example compared to the African cichlid radiations, with a maximum sequence divergence among species in cytochrome *b* (mtDNA) of 18%, or approximately 9 million years (Near and Bernard, 2004). The group therefore comprises a range of divergence times between species and obvious divergence in male nuptial color.

Each species in the subgenus is characterized by a vibrant, species-specific nuptial coloration expressed on the body and fins of males during the breeding season (typically March-May), and the most closely related species are distinguished primarily by these visual traits (e.g., Page, 1983; Porter et al., 2002; Powers and Mayden, 2003). Females have mate preferences based on visual cues. *Etheostoma barrenense* and *E. zonale* are characterized by red and green nuptial coloration, respectively, and females of both species prefer to associate with conspecific over heterospecific males (Williams and Mendelson, 2010). Additionally, sexual (behavioral) isolation evolves faster than hybrid inviability in *Etheostoma*; that is, the first reproductive barrier to arise between diverging lineages appears to be the failure to recognize each other as suitable mates (Mendelson, 2003; Mendelson et al., 2007).

While the exact mechanisms of color production in darters are not yet reported, it is thought that blue and green colors are produced via a different biochemical pathway than red, orange and yellow colors (Gumm et al., in press; Porter, 1999). Red, orange and yellow are generated according to the presence of typically carotenoid based pigments (Grether et al., 2004; Hill and McGraw, 2006) and evidence suggests this is the case in darters as well (Gumm et al., in press; Porter, 1999). The blues and greens of darters also appear to be pigment-based, with green likely being due to overlaying blue and yellow pigments (Gumm et al., in press; Pearsall, unpubl. data). Pigment-based blue and green colors are rare in animals (Bagnara et al., 2007), and the identity of these pigments and their underlying production mechanisms have yet to be identified in darters.

We aimed to examine the evolution of nuptial coloration in darters using a comparative phylogenetic approach. First, we used spectral reflectance to define broad color classes present in these fishes and quantified differences in hue within each color class. Next, we assessed evolutionary patterns within and between different color classes expressed within a body area. Given potentially different underlying mechanisms producing different color classes, we predicted that different color classes evolve independently. Third, we examined the evolution of conspicuous male coloration across different body areas. Darters display two large, conspicuously colored dorsal fins during courtship and aggression and collapse these fins when at rest or foraging, analogous to the dewlap displays of lizards. We therefore tested for differences in the evolutionary patterns of coloration among body regions to determine whether

colors on different regions of the body are evolving in concert or independently. Finally, we tested whether those regions involved in behavioral displays during courtship and intraspecific aggression (i.e. fins) are more evolutionarily labile.

1 Material and Methods

1.1 Collection and maintenance of fishes

We examined 17 species from the monophyletic subgenus *Ulocentra* (Porter et al., 2002). These species include representative samples of the color diversity observed in darters, including red, orange, yellow, green, and blue. Males in breeding condition were collected by seine net in March and April 2009 (Table 1). Fishes were transported by car in aerated coolers to the laboratory at the University of Maryland, Baltimore County. In the lab, fishes were separated by species and housed in 10 L tanks on a re-circulating aquarium system (Aquatic Habitats Inc.). They were kept at a constant temperature of 12–14°C on a 12:12 L:D cycle consisting of fluorescent bulbs in addition to natural light through standard glass windows. Fishes were fed live blackworms once daily.

1.2 Measurement and analysis of reflectance spectra

Reflectance spectra were measured from five adult males of each species as in Gumm et al. (in press). All reflectance spectra measurements were obtained from whole animal specimens immediately after euthanasia with MS-222 (tricaine methanesulfonate). Immersing fish in MS-222 appears to cause maximal color expression in this system and removes potential variation in coloration that may be due to the effects of motivational state (pers. obs). Sampled fishes were pinned onto a non-reflective black background with a diffuse reflectance standard (Spectralon, Labsphere) placed underneath fins, which were pinned fully erect. All fishes were kept wet by repeated application of reverse osmosis water throughout the sampling period.

Reflectance spectra were measured using a spectrometer (Ocean Optics HR2000+) and were illuminated via a full spectrum, deuterium- tungsten halogen light source (Ocean Optics DH-2000). A bifurcated fiber optic cable was used, allowing illumination and detection to occur through the same probe. All measurements were taken at 45° at a distance of approximately 1 mm from the specimen, allowing for measurement of small spots of color approximately 0.1 mm in diameter. We identified the size and precise location of the area sampling through preliminary tests using samples of known size and color. Measurements were taken in a dark room to prevent additional light from influencing the measurement. White and dark standards were obtained at regular intervals, approximately every 5–10 min. White standards were generated from diffuse reflectance standard (Spectralon, Labsphere) and dark standards were generated through an Olympus BX41compound microscope via a 1000μm optical fiber (Ocean Optics) fixed to the microscope viewing head with the shutter to the spectrometer closed, removing all light. Spectra Suite (Ocean Optics) software was used to visualize and store reflectance data files. Each file resulted from an average of 10 scans across all wavelengths (300–900 nm) with an integration time of 7–10 ms. Three sample spectra were generated for each color present on the anterior (spinous) dorsal fin, posterior (soft) dorsal fin, anal fin and body (Fig. 1c). When multiple colors were present within a body area (e.g., dorsal bands, body bars) all colors discernable by eye were sampled.

Studies of the evolution of coloration typically define color traits as discrete characters; however, recent studies have also reconstructed color as a continuous character (i.e. spectral location) derived from reflectance spectra (Hofmann et al., 2006; Prager and Andersson, 2009). For our analysis, we defined color in two ways, as a discrete character that is present or absent in a body area and as a continuous character by quantifying spec-

Table 1   Species sampled and collection locations

| Taxon	Location
E. baileyi	Middle Fork of the Red River, Powell Co., KY
E. barrenense	East Fork of the Barren River, Monroe Co., KY
E. bellator	Blue Spring Creek, Blount Co., AL
E. brevirostrum	Shoal Creek, Cleburne Co., AL
E. cervus	Clark's Creek, Chester Co., TN
E. coosae	Shoal Creek, Cleburne Co., AL
E. duryi	Sweetens Creek, Marion Co., TN
E. etnieri	Cherry Creek, White Co., TN
E. flavum	White Oak Creek, Humphrey Co., TN
E. lachneri	Little Sandy Creek, Tuscaloosa Co., AL
E. pyrrhogaster	Thompson Creek, Weakly Co., TN
E. rafinesquei	Barren Run, Larue Co., KY
E. ramseyi	Schultz Creek, Bibb Co., AL
E. raneyi	Big Spring Creek, Marshall Co., MS
E. simoterum	Spencer Creek, Wilson Co., TN
E. tallapoosae	Buck Creek, Clay Co., AL
E. zonistium	Lick Creek, McNairy Co., TN

tral location of each reflectance curve. When recording spectral reflectance data, a discrete color category (red, orange, yellow, green or blue) was assigned to each sample by the observer. While this is a subjective method, observer classification is common in studies of color (Nicholson et al., 2007; Ornelas et al., 2009; Wollenberg et al., 2009) and Armenta et al., (2008) found no difference in quantifying aspects of sexual dichromatism between this and other methods.

To identify quantitative differences between colors, we further assessed if colors that were discernable to us had differences in the shapes of the reflectance spectra. The shape of reflectance spectra can be characterized by the quantitative variables hue, saturation (or chroma) and brightness (Wyszecki and Stiles, 1982; Endler, 1990). Hue relates to our perception of different colors based on the wavelengths they reflect. Saturation (or chroma) corresponds to how rapidly reflectance intensities change across the spectrum, and brightness corresponds to the overall amount of light reflected. We used the segment classification method of Endler (1992) to calculate 5 measures of chroma for each reflectance curve. Segment classification identifies differences in shape across the spectrum while accounting for differences in brightness (Montgomerie, 2006). A within-species principle component analysis was used to distill the 5 chroma variables into one or two components, which accounted for over 87% of the variation in shape of the spectra. Principle components were plotted to assess any overlap between clusters identified by the observer as different colors. This analysis verified our observations that red, orange and yellow were different color categories as they differed in shape; however, blue and green colors clustered together and therefore were combined into one color category.

Continuous variation in color was quantified within each discrete color category. The variable most closely associated with wavelength reflected is hue; however, as strictly defined, hue depends on the visual system perceiving the colors. Therefore, we assigned a 'spectral location' for each reflectance spectrum based on the shape of the reflectance curve that is independent of the visual system (Andersson and Prager, 2006; Montgomorie, 2006).

Spectral reflectance measurements of blue and green colors often have a characteristic shape consisting of a unimodal peak in the visual part of the spectrum (400–700 nm; Marshall, 2000; Fig. 1a), and the spectral location for these spectra was calculated across the spectrum (300–800 nm) as the wavelength of maximum reflectance of this peak (λ_{max}). Calculations used custom macros in Excel to optimize the fit between a standard curve and the data and then calculate the wavelength corresponding to the peak of the optimized curve.
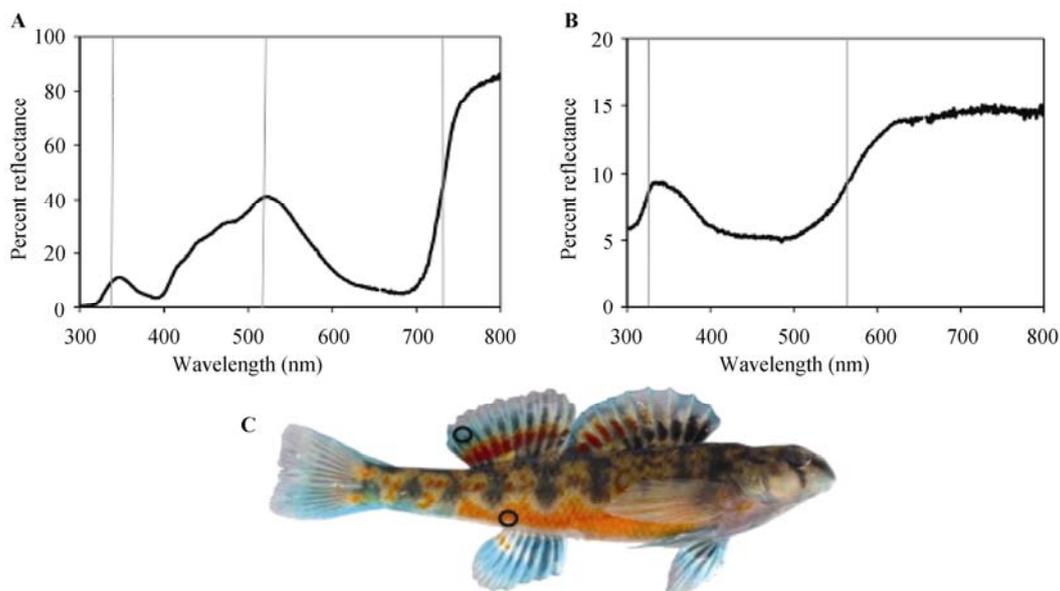
Fig. 1 Sample reflectance spectra of darter nuptial coloration
A. Mean reflectance of blue/green color found on the posterior dorsal fin. The middle grey line corresponds to the peak reflectance, measured as spectral location. Additional grey lines identify the peak reflectance of the UV component and the reflectance midpoint of the far-red component of the blue/green coloration. **B**. Mean reflectance of orange coloration found on the body. Grey lines correspond to the wavelength of midpoint reflectance of the step-shaped curve and the peak of the UV component of the coloration. **C**. Male *E. lachneri* with breeding coloration. Black circles correspond to areas sampled to generate curves seen in (A) and (B).

Orange, red and yellow colors generally exhibit a characteristic step-function shape (Marshall, 2000; Fig. 1b). To assign a spectral location to the orange and red spectra, we used custom software to determine baseline values of minimum and maximum reflectance and calculated the wavelength corresponding to the reflectance midpoint between the maximum and minimum reflectance (λ_{R50}) across the spectrum (300–800 nm). While colors in all color categories did reflect in the ultraviolet portions of the spectrum, λ_{max} and λ_{R50} were always found to be between 400–700 nm.

1.3 Comparative phylogenetic methods

Comparative analyses were conducted using discrete and continuous color variables. For discrete variables, each color was scored as present/absent for each body area. Species were scored as having a color present in a body area if at least two individuals expressed the color there. Within each color, spectral location values were treated as continuous variables. We first determined the best-fit model of evolution for all characters. For discrete characters, two models (equal rates and all rates different) were tested for fit using the fitDiscrete function in the geiger package (Harmon et al., 2008) in R version 2.11.1 (R Core Development Team, 2010). The equal rates model estimates the rate of transition between states (i.e. gains or losses) to be equal, whereas the all rates different model estimates asymmetric rates of transition (Pagel,

1994). To test for significance, we generated a p value by comparing the results of a likelihood ratio test to a chi-squared distribution. When there was no significant difference between models, the single parameter equal rates model was designated as the most appropriate model. In consequent analyses, the best-fit model was used in all cases. For continuous characters, five phylogenetic models of evolution (Brownian motion, early burst, Pagel's lambda, kappa, delta, and Orstein-Uhlenbeck) were tested using the fitContinuous function in the geiger package in R. An Akaike Information Criteria (AIC) test then assessed the likelihood values for the best-fit model.

We performed ancestral state reconstruction of all variables onto a previously published AFLP phylogeny of darters (Mendelson and Wong, 2010; Fig. 2). The tree was ultrametricized with the mean path lengths method using the chronoMPL function in the ape package in R. Discrete character states were reconstructed using maximum likelihood methods in Mesquite (Maddison and Maddison, 2001). The continuous values of spectral location were reconstructed using the ace function in the ape package and the getAncStates function in the Geiger package in R. Methods for reconstruction of continuous characters do not allow for missing data, therefore species that did not express a particular color in a particular body area were excluded from the reconstruction for that color/body area.
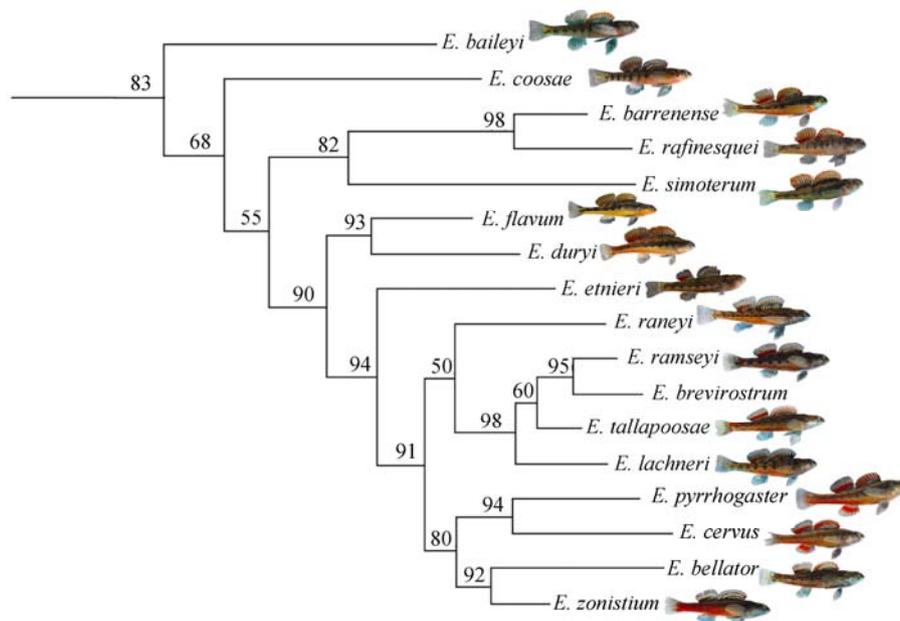
Fig. 2 Neighbor joining cladogram
Adapted from Mendelson and Wong (2010) showing species relationships and male nuptial coloration in snubnose darters. Numbers at nodes are Bayesian posterior probability values.

To determine the extent that variation in color traits is explained by phylogeny, we tested whether each trait showed significant phylogenetic signal. Phylogenetic signal for discrete traits was tested using Pagel's lambda transformations. This method uses maximum likelihood optimization to test how well the data fits trees that are transformed with varying values of the lambda statistic, which correspond to the amount of phylogenetic patterning in the tree (Pagel, 1999). Using the phylosignal function in the picante package in R, we performed a randomization test and generated Blomberg's K statistic to assess the strength of phylogenetic signal for spectral location (Blomberg et al., 2003). Blomberg's K statistic compares the observed measure of phyogenetic signal with an expected measure based upon the topology of the tree and a Brownian motion model of evolution. Values of K near 1 indicate similarity between the observed and expected values, whereas values of K >1 indicates that closely related taxa have traits values more similar than expected and K<1 indicates closely related taxa have trait values less similar than expected, i.e., weak phylogenetic signal.

We tested whether colors were evolving independently or in concert between body areas by testing for co-evolution of color traits. For discrete characters, we assessed correlations in character state changes (present or absent) using Pagel's correlation method (Pagel, 1994) implemented in Mesquite. Pagel's test was run with 10 iterations to optimize the likelihood estimation and p values were calculated from 1000 simulated replicates.

Methods for testing correlations between continuous characters do not allow for missing data, therefore species that did not express a color in both body areas of interest were excluded from the correlation analysis of spectral location. Given that taxon sampling can influence comparative methods (Garland et al., 1993), we did not analyze correlation in spectral location between blue/green colors on the anterior and posterior dorsal fins, or orange on the anal fin, due to these colors being present on these body areas in less than half of the species sampled. Phylogenetic generalized least squares models were run using the nlme and ape packages in the statistical program R to test for significant correlations between color characters found in different body areas. Each model was run with an Ornstein-Uhlenbeck model of evolution based on the outcome of our model fitting tests (Hansen, 1997; Martins and Hansen, 1997; Butler and King, 2004).

2   Results

2.1   Color quantification

We identified 5 main colors found on male darters. Blue and green colors exhibited a unimodal peak whereas red, orange and yellow colors exhibited a step-shaped curve. There was no difference in the shape of the reflectance curve between spectra identified as blue or green and there was continuous variation in the spectral location values for blue/green colors (unpub. data). Thus, "blue" and "green" colors were grouped together as a single discrete character. By contrast, analysis of chroma values for red, orange and yellow colors found significant differences in chroma (unpub. data) indicating that each differs in spectral shape. Therefore, we characterized red, orange and yellow as discrete variables.

Of the four color classes, only orange was reliably found on the body below the lateral line, therefore orange is the only body color reconstructed. Blue/green was found below the lateral line in some individuals, typically associated with melanophores, but quantifiable spectra were extremely rare. Yellow was also present on the body in two species, but in spots too small or diffuse to reliably quantify. Red was not observed on the body below the lateral line. All four colors were found on the anterior and posterior dorsal fin and therefore, all were reconstructed onto the phylogeny. All species exhibited blue/green on the anal fin and some species also expressed orange in the anal fin. Orange on the anal fin was reconstructed onto the phylogeny but blue/green was not due to it being invariant. Red and yellow on the anal fin were rare and could not be quantified reliably.

2.2   Model testing

For most discrete color traits, there was no significant difference in fit between the equal rates and the all rates different models (Anterior dorsal: Red, $P = 0.36$; Orange, $P = 0.05$; Yellow, $P = 0.69$; blue/green, $P = 0.18$; Body: Orange, $P = 0.34$; Posterior dorsal: Red, $P = 0.09$; Orange, $P = 0.23$; Yellow, $P = 0.14$; Blue/green, $P = 0.35$). Thus, the equal rates model, equivalent to the MK1model in Mesquite, was used in subsequent analyses. The all rates model was significantly better for the orange color on the anterior dorsal. The best fitting model for all continuous traits of spectral location values was the Ornstein-Uhlenbeck model.

2.3   Phylogenetic signal

When colors were treated as discrete characters, none of the color traits had significant phylogenetic signal

(Anterior dorsal: Red, $P = 0.16$; Orange, $P = 0.99$; Yellow, $P = 0.26$; blue/green, $P = 0.61$; Body: Orange, $P = 0.21$; Posterior dorsal: Red, $P = 0.63$; Orange, $P = 0.85$; Yellow, $P = 0.99$; Blue/green, $P = 0.29$; Anal fin, Orange, $P = 0.99$). With values of spectral location treated as continuous characters, all body regions had values of Blomberg's K that were < 1, indicating that color traits were less similar among closely related taxa than expected by chance. Only two characters, orange coloration found on the body and blue coloration found on the anterior dorsal fin, exhibited significant phylogenetic signal (Table 2).

2.4 Ancestral state reconstruction

When color traits were reconstructed as discrete variables, patterns of gains and losses varied across colors and across body regions. Colors inferred as present in the most recent common ancestor of sampled species are orange body coloration and all four colors on the anterior dorsal fin (Fig. 3; Fig. 4). All character state changes on the anterior dorsal fin and body resulted from losses of coloration, i.e., there were no independent gains of coloration on these two body regions (Fig. 3; Fig. 4). On the posterior dorsal fin, red and yellow were inferred as present in the common ancestor while blue/green was reconstructed as absent (Fig. 4). Reconstruction of orange on the posterior dorsal fin must be interpreted with caution due to high levels of uncertainty in the reconstructed nodes (Fig. 3). Losses of coloration on the posterior dorsal fin, as on the anterior dorsal fin, were more common than gains, with losses of red, yellow and blue colors (Fig. 4). Additionally, yellow and orange may have been gained in the posterior dorsal fin in some lineages, although there was uncertainty

associated with the nodes indicating potential gains of these colors (Fig. 3; Fig. 4). Two gains and multiple evolutionary reversals of the blue/green coloration were also inferred for the posterior dorsal fin (Fig. 4). All species have blue/green on the anal fin, thus its reconstruction was uninformative. Ancestral state reconstructions of continuous values of spectral location yielded overlapping 95% confidence intervals for all nodes. These values were therefore uninformative and are not presented here.

2.5 Co-evolution of color

Patterns of co-evolution when colors were defined as discrete traits were dependent on where the color was located. No significant correlations were detected between the presence/absence of orange on the body and orange on any of the fins (body-anterior dorsal, $P = 0.634$, body-posterior dorsal, $P = 0.369$, body – anal fin, $P = 0.156$; Fig. 3). However, presence/absence of coloration was significantly correlated across the anterior and posterior dorsal fins (Fig. 4). For colors on the fins, Pagel's discrete model test (Pagel, 1994) resulted in greater log likelihoods for models that account for co-evolution of traits than models for independent evolution of traits. Additionally, simulations indicate that models of correlated evolution are significantly better than models of independent evolution (anterior-posterior dorsal red: $P = 0.033$; anterior-posterior dorsal orange: $P = 0.032$; anterior-posterior dorsal yellow: $P = 0.007$, anterior-posterior dorsal blue/green: $P = 0.020$; anterior dorsal-anal fin orange: $P = 0.046$). The rate parameters generated from the simulations indicate that the probability of loss of a color is much higher than the probability of gain.

Table 2 Phylogenetic signal of color traits

	Trait	K statistic	P value
Anterior Dorsal Fin	Red	0. 6847257	0.203
	Orange	0. 6986548	0.298
	Yellow	0. 5859465	0.501
	Blue/Green	1.013411	**0.024**
Body	Orange	0. 9437748	**0. 046**
Posterior Dorsal Fin	Red	0. 6535494	0.386
	Orange	0. 4512931	0. 858
	Yellow	0. 5328395	0.688
	Blue/Green	0. 6661143	0.326
Anal Fin	Blue/Green	0. 6051258	0.34

Blomberg's K statistic (Blomberg et al. 2003) is listed for coloration among body areas. *P* values are generated using randomization tests. *P* values are generated using randomization tests.
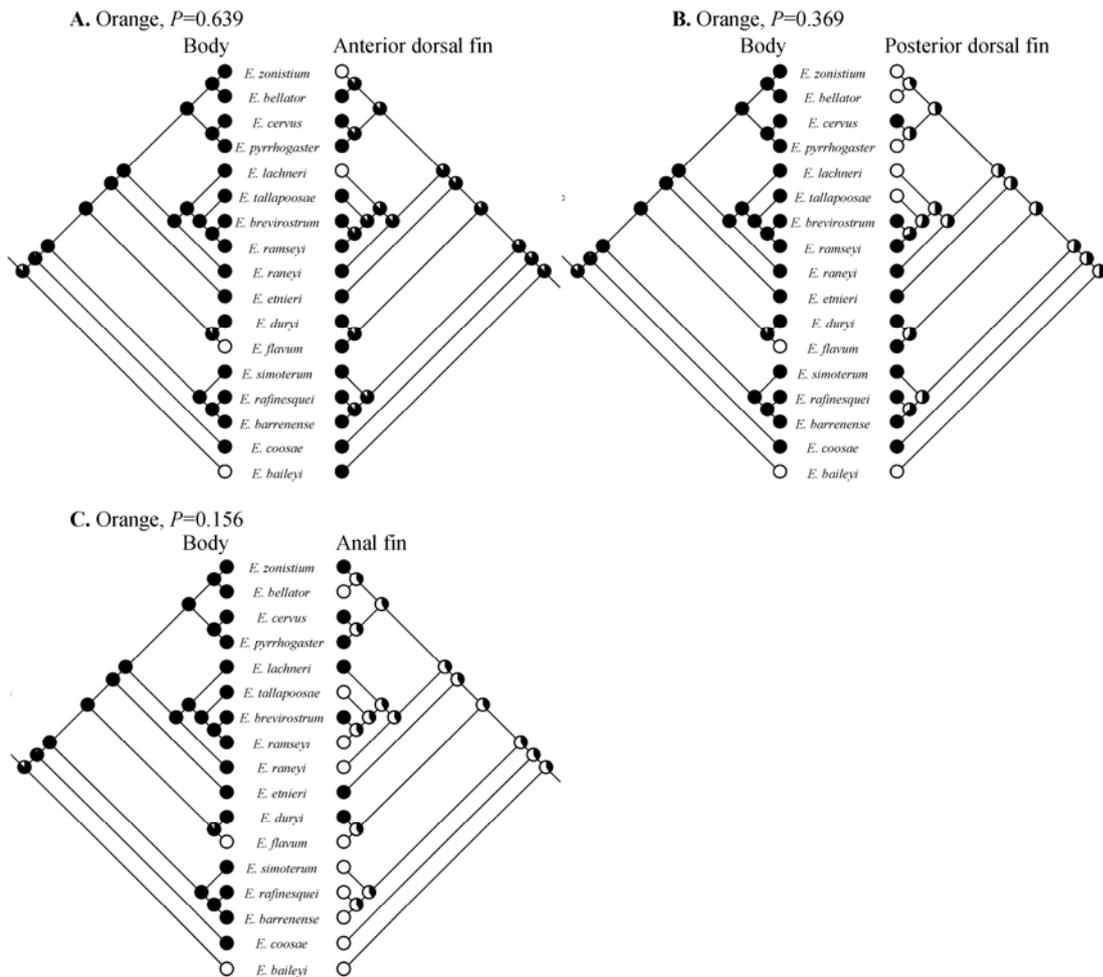
Fig. 3   Ancestral state reconstructions showing the distribution of orange coloration found on the body and fins across species of darters

Black circles indicate presence of coloration, and white circles indicate absence of coloration. Nodes represent likelihood values of the reconstructed ancestral state. *P* values are from analyses of correlated evolution on discretely coded characters.

No significant correlations were detected between continuous values of spectral location of color on the body and spectral location of colors on the fins (PGLS; Table 3). Significant correlations were found, however, between spectral location on the anterior and posterior dorsal fins for orange coloration, and a strong but non-significant trend was found for correlated evolution of spectral location of red and yellow between the anterior and posterior dorsal fins (PGLS; Table 3). We also found a strong but nonsignificant correlation between spectral location of blue/green coloration on the anterior dorsal fin and the anal fin (PGLS; Table 3).

3   Discussion

3.1   Darter colors

Nuptial coloration of darters differs among species both in the colors present in particular body areas and in quantitative aspects of spectral reflectance. Our analyses of spectral location and chroma indicated that there are four discrete color classes in darters: red, orange, yellow and blue/green. Within each of these color classes, spectral location values are continuously distributed. The most comprehensive studies of fish coloration to date identify 21 broad color categories based on a qualitative assessment of the general shape of the spectral reflectance curve (Marshall, 2000; Marshall et al., 2003). Based on these general shapes, darter colors appear most similar to the Red-UV, Orange-UV, Yellow-UV, and the "Labriform" categories identified by Marshall, corresponding to darter red, orange, yellow, and blue/green, respectively.

Some components of the spectral reflectance curves used by Marshall (2000) to define color categories were not quantified in the current study. For example, all
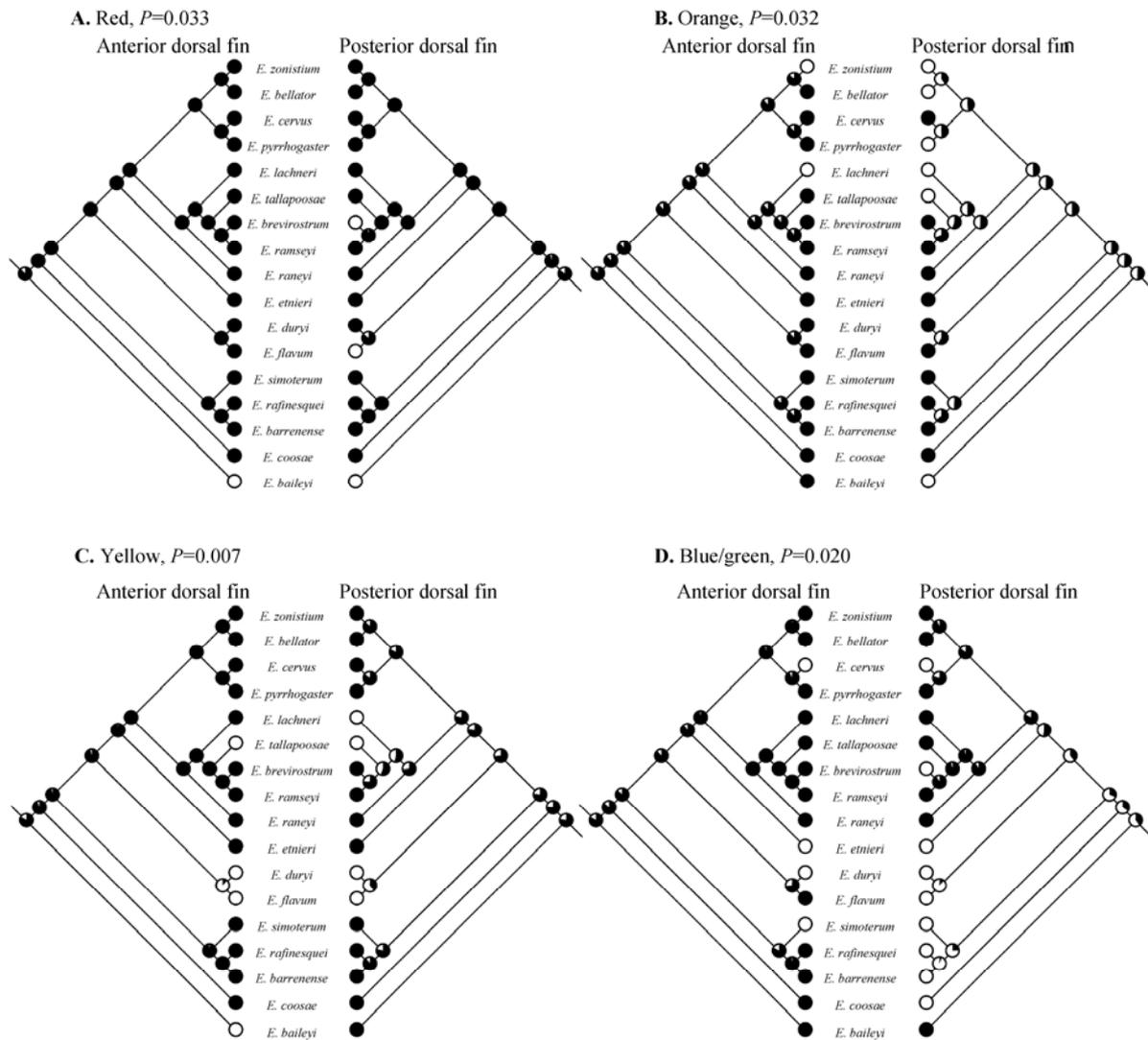
Fig. 4 Ancestral state reconstructions showing the distributions of colors found on the anterior and posterior dorsal fins
Black circles indicate presence of coloration, and white circles indicate absence of coloration. Nodes represent likelihood values of the reconstructed ancestral state. P values are from analyses of correlated evolution on discretely coded characters.

Table 3   Co-evolution of coloration across body areas

		Anterior Dorsal Fin											
		Red			Orange			Yellow			Blue/Green		
		β	α	P	β	α	P	β	α	P	β	α	P
Body	Orange	0.2655	5.7585	0.2487	−0.8095	75.0964	0.1081	−0.0572	185.3010	0.7239	−0.0213	0.8126	0.9696
Posterior Dorsal Fin	Red	0.5470	3.2574	0.0587									
	Orange				0.8347	9.42e-07	0.0333						
	Yellow							0.6956	40.7517	0.0575			
Anal Fin	Blue/Green										0.8116	6.2965	0.0632

Correlation coefficient, alpha parameter and P values from PGLS analysis

colors expressed in darters (red, orange, yellow, green and blue) can have an ultraviolet component to their spectral reflectance (Fig. 1). The role of UV signaling in this system remains unknown, and preliminary analyses of visual sensitivity suggest darters lack a UV sensitive visual pigment (unpublished data). Spectral location of the UV component of reflectance curves therefore was not analyzed in the current study.

The blue/green colors of darters also have a step-shaped component in the far-red region of the spectrum (Gumm et al., in press; Fig. 1). While species may differ in the spectral location of this step component (Gumm et al., in press), the function of this reflectance is unknown, and it is likely outside the range of fish visual sensitivity. Spectral location of the far-red component of the blue/green colors therefore was not included in our analyses, though Marshall (2000) uses this component as a defining feature of the "labriform" color categories. Interestingly, the labriform categories are rare among reef fishes, being found only in parrotfishes and wrasses (Marshall et al., 2003). Given that blue and green colors in darters appear to be pigment based (Pearsall, unpubl. data), rather than structural, as in most vertebrates (Bagnara, et al., 2007), it would be interesting to determine the mechanisms producing labriform colors in reef fishes.

We also note that the color classes identified in this study differ slightly from conclusions of Gumm et al. (in press) in which blue and green colors were analyzed from four species and were treated as separate color classes. The present classification is based on analysis of chroma and spectral location for most species of *Ulocentra*, thus the broad differences in spectral location of blue and green color traits in the previous study were likely due to limited taxon sampling.

3.2   Phylogenetic signal

Coloration in darters appears to be highly labile. No significant phylogenetic signal was detected for colors as discrete traits. When color was reconstructed as a continuous character (spectral location), most colors in most body regions yielded values of the K statistic <1 and did not show significant phylogenetic signal. Significant phylogenetic signal was found only for the orange body color and the blue/green color on the anterior dorsal fin. Evidence suggests that most traits, with the exception of body size, exhibit less phylogenetic signal than expected (Blomberg et al., 2003), and our data fit this general pattern. A similar comparative study in birds, which used spectral location to map color also found low values for the K statistic; however, these values still resulted in significant phylogenetic signal (Hofmann et al., 2006). Typically, the K statistic has been interpreted to indicate evolutionary lability and deviation from Brownian motion (Blomberg et al., 2003). Many studies also use phylogenetic signal to indicate the rate and/or process of evolution acting on a character (Blomberg et al., 2003; Parra, 2010). Recent theoretical work, however, suggests that values of phy-

logenetic signal *per se* should not be used to infer rate or evolutionary process (Revell et al., 2008). Given this, we simply infer that there are differences in evolutionary lability between coloration on the body as compared to the fins, and we hypothesize that this may result from distinct evolutionary pressures.

Another explanation for the low phylogenetic signal observed in color traits is that color may be influenced by the environment, and relevant environmental variables are not phylogenetically conserved. In particular, colors based on carotenoids, which must be acquired from the diet, have been shown to be condition dependent in numerous taxa (Fujii, 1969; Kodric-Brown, 1989; Hill and McGraw, 2006). However, male nuptial coloration in darters appears to have a genetic basis, as species-specific differences in carotenoid composition have been observed in common garden experiments in *Ulocentra* (Porter, 1999). Moreover, we observed significant phylogenetic signal in the main body color, orange, which is likely a carotenoid based color. We therefore interpret the low phylogenetic signal observed in most color traits as evidence of evolutionary lability.

3.3   Ancestral character states

Ancestral character state reconstruction of spectral location yielded overlapping confidence intervals across all nodes for all color classes and was therefore uninformative. Character state reconstruction of colors as discrete traits (presence/absence), however, was comparatively instructive. On the body, the presence of orange appears to be ancestral in this group, being lost in only two species. *Etheostoma flavum*, the saffron darter, and *E. bailey*, the emerald darter, do not exhibit orange on the body, but both exhibit yellow body coloration. The adaptive significance of the loss of orange is unclear, though Etnier and Bailey (1989) point out that the substrate in the habitat of *E. flavum* is dominated by yellow-orange rocks; whereas, its orange-colored sister species *E. duryi* is found in habitats with darker substrates. The underlying mechanisms producing orange and yellow coloration are also unknown, though red, orange and yellow colors have been identified as carotenoid-based in some darter species (Porter, 1999; Smith and Mendelson unpub. data) and are inferred as such in other species based on the shape of their spectral reflectance curve (Gumm et al., in press). Identifying the biochemical basis of pigmentation in darters would allow us to determine, for example, if losses of orange and/or gains of yellow on the body were due to different underlying mechanisms or changes in a similar pigmentation pathway.

Reconstructing the presence or absence of color classes on the fins suggests that, like orange body color, most colors on the fins were present in the most recent common ancestor. It is important to note, however, that we scored colors as 'present' if at least two sampled males expressed the trait; therefore, like some extant species, the ancestor may have been polymorphic for a given color on the fin, or simply possessed the ability to express the trait. Discrete reconstruction of fin colors also suggests that patterns of color evolution differ between the anterior and posterior dorsal fin. Specifically, there were more losses of color on the posterior dorsal fin than on the anterior dorsal fin, and this pattern was seen for all colors.

The loss of elaborate traits is well established from comparative and empirical studies (reviewed in Wiens, 2001). Given that sexual selection is likely involved in the evolution and maintenance of these colors, their loss across all body areas may be due to stronger, competing selective pressures. For example, strong natural selection can oppose sexual selection and thus contribute to the loss of color ornaments. In guppies *Poecilia reticulata*, variation in predation pressure influences male coloration such that less colorful males are associated with greater predation pressure (Endler, 1983). Alternatively, the strength of sexual selection may vary across lineages or even reverse over time, potentially leading to the loss of an elaborate trait (Morris et al., 2005; Rosenthal et al., 2002). Addressing hypotheses of color loss in darters, including environmental, behavioral or random factors (reviewed in Wiens, 2001) will require additional comparative and experimental studies.

3.4   Co-evolution of coloration across body areas

Relationships among colors depended on their location on the body. That is, colors appear to have co-evolved across some, but not all body areas. The presence or absence of orange on the body was not correlated with the presence or absence of orange on any of the fins. However, presence/absence of orange on the anterior and posterior dorsal fins was correlated. We also found no significant correlations between the spectral location of body colors and fin colors. However, we found significant correlations between spectral location values of colors on the anterior and posterior dorsal fins, as well as correlations between the anterior dorsal and anal fin. In some cases, the correlations among colors on the fins represent statistical trends; however, the near significance is notable given the degree of variation in spectral location found within species. Together, correlation analyses of both discrete and continuous measures of color therefore suggest that coloration on the body is evolving independently of coloration on the fins, and that coloration on the fins is evolving in concert.

The patterns of covariance among body areas may indicate the functional hypothesis underlying the evolution of complex coloration in darters. Correlations among coloration on the fins could suggest that these signal components are redundant and convey the same information (Moller and Pomiankowski, 1993; Johnstone, 1997). The fins are typically raised and lowered together, further suggesting they may have a similar function. Alternatively, correlation among the fins may be due to a physiological constraint that prevents independent evolution of fin colors (Hebets and Papaj, 2005). The lack of covariation in coloration between the body and fins suggests that coloration on the body may function to convey different information than coloration on the fins (e.g. multiple messages, Moller and Pomiankowski, 1993; Johnstone, 1997). These results also suggest that selection pressures for coloration may differ between the body and the fins and that evolution of nuptial coloration may be influenced by display behavior. We cannot rule out alternative functional hypotheses based on our results; however, the observed patterns of color evolution among body areas may inform future research examining the function of each color component.

In darters, the dorsal fins and anal fin are used in courtship and aggressive interactions between conspecifics. While the flank of the body is always displayed, dorsal and anal fins are erected and retracted during conspecific interactions, potentially allowing a private communication channel between individuals. A private channel of communication would be inferred if the fins are not raised in the presence of predators, for example, or if predators are unable to detect the colors expressed on the fins. Swordtails *Xiphohphorus nigrensis* use UV reflectance as a private channel of communication, as females prefer males with more UV and one of the main predators is relatively insensitive to UV (Cummings et al., 2003). If male fin color represents a private channel of communication in darters, it remains unclear whether these signals are intended for primarily male or female receivers. Male fin displays are directed at both females during courtship and at other males during aggressive interactions. Future empirical work would be necessary to determine whether inter- and intra-sexual selection pressure act independently or interact to influence the evolution of coloration on the fins.

3.5 Evolutionary implications of multi-component signaling

Many models to explain the evolution of multicomponent traits have been proposed (Lande, 1979; Lande and Arnold, 1983; Hebets and Papaj, 2005) and supported empirically (Zuk et al., 1990; Omland, 1996; reviewed in Candolin, 2003), but fewer studies examine the evolutionary implications of such traits (but see Hohenlohe and Arnold, 2010). The evolution of multicomponent traits may have broad implications for speciation, as divergence in sexually selected traits leading to behavioral isolation provides a direct link between sexual selection and speciation (Lande, 1981). When traits with multiple components are sexually selected, each component of the trait may play a role in mate choice (but see Omland, 1996). For example, Uy and Borgia (2000) examined how different components of male bowers influenced female choice between two populations of the Vogelkop bowerbird *Amblyornis inornatus*. Males from the two populations differed in multiple trait components relating to bower structure and bower decoration, and females used multiple cues of color and size to assess male quality and choose mates (Uy and Borgia, 2000). Ultimately, the co-evolution of multiple signals and multiple preferences may accelerate the evolution of behavioral isolation between these two populations.

How multicomponent traits influence speciation also may depend on the model of sexual selection. Under the sensory drive model, environmental variation causes divergence in female sensory systems, which in turn create novel selection pressures driving the evolution of signals (Ryan, 1990; Endler, 1992). The evolution of nuptial coloration in fishes in particular has been examined in the context of sensory drive. In a number of systems, quantitative measures of coloration, visual sensitivity and environmental light variables are correlated between populations or closely related species, supporting a sensory drive model of divergence (Boughman, 2001; Fuller et al., 2005; Cummings, 2007; Seehausen et al., 2008). Notably, these studies examine only one component of coloration (but see Cummings, 2007). If sensory drive is operating in darters, and if, as our results suggest, color traits are evolving independently in different body areas, then only certain traits would be predicted to correlate with visual sensitivity and environmental variables. Which traits these may be remain to be determined; however, females of at least one pair of darter species significantly prefer both the main body color and patterning elements of conspecific males

(Williams and Mendelson, in review).

Like many lineages, darters show striking differences in complex male signals among closely related species, and the causes and consequences of this divergence form the basis of ongoing research. Here, we showed that most, but not all, components of darter coloration exhibit high evolutionary lability and that distinct components of these complex signals may be evolving independently. The observed patterns support the hypothesis that different components of complex signals may be subject to distinct evolutionary pressures, and more specifically that the combination of behavioral displays and morphology in communication may have a strong influence on patterns of signal evolution.

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