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Divergence of Threespine Stickleback That Differ in Nuptial Coloration

Abstract

Recent research has led to a much better understanding of the evolutionary processes that mold and structure variation within and among populations. How populations diverge at the genome-wide level and how polymorphism is maintained within a species, however, remains unclear. We address these questions with two freshwater color morphs, red and black, of the threespine stickleback fish (*Gasterosteus aculeatus*) from the northwest United States, in which a shift from red to black nuptial coloration occurred in several locations following glacial retreat. We measured phenotypic variation in a suite of traits and used next generation sequencing to characterize within-species and among-morph genetic variation between the two morphs. We found substantial phenotypic and genetic divergence between color morphs, and patterns observed in a third, "mixed" morph that are likely due to hybridization between anadromous and freshwater stickleback. This work highlights the central role of natural and sexual selection in the evolution of divergence in nature.

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Divergence of Threespine Stickleback That Differ in Nuptial Coloration

A Thesis

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Clara Sophie Jenck

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Advisor: Dr. Robin M. Tinghitella

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Abstract

Recent research has led to a much better understanding of the evolutionary processes that mold and structure variation within and among populations. How populations diverge at the genome-wide level and how polymorphism is maintained within a species, however, remains unclear. We address these questions with two freshwater color morphs, red and black, of the threespine stickleback fish (*Gasterosteus aculeatus*) from the northwest United States, in which a shift from red to black nuptial coloration occurred in several locations following glacial retreat. We measured phenotypic variation in a suite of traits and used next generation sequencing to characterize within-species and among-morph genetic variation between the two morphs. We found substantial phenotypic and genetic divergence between color morphs, and patterns observed in a third, “mixed” morph that are likely due to hybridization between anadromous and freshwater stickleback. This work highlights the central role of natural and sexual selection in the evolution of divergence in nature.

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Chapter 1: Phenotypic divergence among threespine stickleback that differ in nuptial coloration

Introduction

Much of the historical work on the origin and maintenance of biodiversity has relied heavily on the characterization of phenotypic variation as a basis for inferring the existence and trajectory of evolutionary change (Darwin 1859; Wallace 1870). The substantial variation in traits we observe among taxa supports the hypothesis that divergent selection can drive reproductive isolation, which builds as a result of adaptation to contrasting selection regimes imposed by different environments (Schluter 2001). Both natural and sexual selection are important evolutionary forces that can generate and shape phenotypes, and also have roles to play in the generation of biodiversity (speciation; Ritchie 2007; Safran et al. 2013; Servedio and Boughman 2017).

Divergent natural selection among populations can arise because of differences in habitat, resources, climate, and predation (Schluter 2001). In three lizard species that inhabit the White Sand dunes in New Mexico, for instance, cryptic coloration has rapidly evolved and is selectively maintained by predation, relative to their background environment (Rosenblum et al. 2010). Environmental differences can also affect sexually selected traits through interactions with eavesdropping predators and parasites (reviewed in Zuk and Kolluru 1998), interspecific (reviewed in Groening and Hochkirch 2008) and intraspecific competing signalers (reviewed in Tinghitella et al. 2018b), and transmission properties of the environment (Endler 1992; Boughman 2002; Seehausen et al. 2008), placing sexually selected traits under conflicting selection that shapes phenotypic and genetic variation within and among populations. For example, natural selection imposes a cost on conspicuous sexual displays, such as in the Pacific field cricket (*Teleogryllus oceanicus*) where male calling song also attracts parasitoids (Zuk et al. 2006) and

environmental conditions affect the transmission and perception of colorful sexual signals in guppies (*Poecilia reticulata*; Endler 1991). Recent work has emphasized how natural and sexual selection work jointly to drive evolutionary change, divergence, and even speciation (Safran et al. 2013). Here, we measure phenotypic change in a suite of traits across several populations of fish that have undergone recent divergence in their sexual signals, that likely stem from habitat variation.

Species that have diversified over relatively short time scales and that are distributed across landscapes with varied environmental characteristics likely to generate divergent selection shed important light on the evolutionary processes underlying phenotypic change. The threespine stickleback (*Gasterosteus aculeatus*) is one such model study system. These fish episodically colonized freshwater habitats from marine environments following glacial retreat at the end of the Pleistocene epoch less than 12,000 years ago (McPhail 1994). In many cases, the resulting freshwater populations have diverged phenotypically from marine ancestors in parallel ways, offering natural, replicated, and independent evolutionary experiments. In colonizing freshwater habitats, stickleback experience selection that leads to divergence in color, shape, size, salinity tolerance, and foraging behavior and morphology (reviewed in McKinnon and Rundle 2002). Stickleback populations have also undergone divergence in the presence and number of lateral bony plates, a trait that has quite famously evolved repeatedly and predictably in response to freshwater-marine differences in predation and salinity (Bell 2001; Reimchen and Nosil 2004; Marchinko and Schluter 2007). Typically, marine fish are larger and have fully plated bodies whereas stream-dwelling freshwater populations are smaller and tend to have low or partially plated morphs (Hagen and Gilbertson 1973; Bell and Foster 1994).

Though there are several parallels in the divergence of freshwater and marine stickleback, sexually selected traits have also undergone rapid evolutionary change in freshwater stickleback populations. Like ancestral marine stickleback, male stickleback from most derived freshwater populations display a bright red throat during the breeding season (hereafter referred to as red stickleback; McPhail 1969; Semler 1971; Hagen and Moodie 1979). However, in several

locations along the Pacific coast of North America, males have lost their iconic mating signal, and instead have full-body black breeding coloration (hereafter referred to as black stickleback; McPhail 1969; Semler 1971; Reimchen 1989; Boughman 2001). The prevailing explanation for this evolutionary switch is sensory drive, the process by which sexual signals shift to improve transmission in their environment (Endler 1992; Boughman 2002). Red stickleback are often found in habitats with relatively clear water whereas black stickleback are found in red-shifted, tannin-rich waters, making males of each color morph highly contrasted and more visible to the drab females in their respective environments (Reimchen 1989; Scott 2001; Boughman 2001). Boughman et al. (2001) show that in red (limnetic - relatively clear water) and black (benthic - relatively red-shifted water) British Columbian stickleback from Lake Paxton and Lake Enos, the extent of divergence in male color and female preference for male color is correlated with the extent of reproductive isolation among populations, supporting a role for sensory drive in speciation.

Recent work in red and black stickleback from Washington State similarly supports a role for sexual selection in divergence of red and black stickleback, albeit through a different mechanism of sexual selection (Tinghitella et al. 2015; Tinghitella et al. 2018a). In simulated secondary contact in the laboratory, females from populations containing only red or only black males retain their ancestral preference for the red mating signal (McKinnon 1995) and prefer to interact with red males (Tinghitella et al. 2015). Though there is no evidence of assortative mating, male competition for territories, which occurs prior to female mate choice in the breeding season, may be a more important isolating mechanism in this system; black males bias their aggression towards red males, which may contribute to habitat and reproductive isolation between the two color morphs (Tinghitella et al. 2018a).

In this study, we measure a comprehensive suite of phenotypic traits that have evolved in parallel in freshwater stickleback (McKinnon and Rundle 2002) including nuptial color, shape, size, and body armor in Washington populations of red and black stickleback. Several of these traits are correlated and possibly genetically linked to one another, so the recent and rapid

changes in color may be associated with changes in a suite of traits that are associated with reproductive isolation in this system. For instance, body shape is correlated with male nuptial color such that deeper bodied fish have redder throats (Malek et al. 2012), suggesting possible linkage of the two. Additionally, several studies have implicated a role for body size and shape in the adaptive divergence of stickleback and as a driver of prezygotic reproductive isolation through size-assortative mating (McPhail 1977; Nagel and Schluter 1998; Head et al. 2013).

Unveiling when or how traits are selected for or against is key to understanding the patterns of phenotypic variation observed in natural populations and assessing variation in locations where multiple morphs coexist and possibly interbreed can offer even more insight into the processes that maintain biodiversity (Schluter 2000; Hoekstra et al. 2004; Roulin 2004; Rueffler et al. 2006; Gray and McKinnon 2007). In pioneering work, McPhail (1969), and Hagen and Moodie (1979), found a region in southwest Washington, Connor Creek, where both red and black stickleback were found with overlapping breeding areas and seasons. Our own surveys in 2018, revealed a site with only black fish plus locations where males had apparent continuous variation in color that prevented us from characterizing fish as clearly red or black. If red and black stickleback interbreed within Connor Creek, we may find a phenotypic cline indicating the presence of a hybrid zone or localized adaptation to an environmental gradient (Endler 1977). We surveyed phenotypic divergence of stickleback across six sites where red and black fish are allopatric (non-overlapping in distribution) and also take a finer-scale approach by examining the phenotypic divergence of color morphs where they historically co-occurred in a single location. Given the variation in nuptial color between morphs, the correlated evolution of shape and color (Malek et al. 2012), habitat differences, and the parallel evolutionary loss of plating in freshwater stickleback across the northern hemisphere, we expect red and black color morphs to differ in body shape, size, and plating, in addition to color. To our knowledge, this is the first in depth investigation of variation in morphological traits (aside from coloration) in WA populations of red and black stickleback.

Methods

Sample collection

We collected sexually mature, adult stickleback from streams and rivers of southwest Washington, US, and transferred them to the University of Denver during the summers of 2013-2015 (Figure 1.1A). Fish with red nuptial coloration were collected from two sites (Campbell Slough (R1) and Chehalis River (R2)) where black fish are not found, and fish with black nuptial coloration were collected from four sites (Vance Creek (B1), Black River (B2), Scatter Creek (B3), and Connor Creek (B4)) where red fish are not found. In summer 2018, we collected stickleback along a 3.5 km transect in Connor Creek, where both color morphs have historically coexisted (McPhail 1969; Hagen and Moodie 1979). To parallel the sampling first done by McPhail (1969), we sampled five locations by paddleboarding along the transect, trapping at approximately 0.9-kilometer intervals, beginning near the mouth of the creek (M1) and moving further inland toward our 2015 Connor Creek sampling site where only black fish are found (B4; Figure 1.1B).

Figure 1.1.

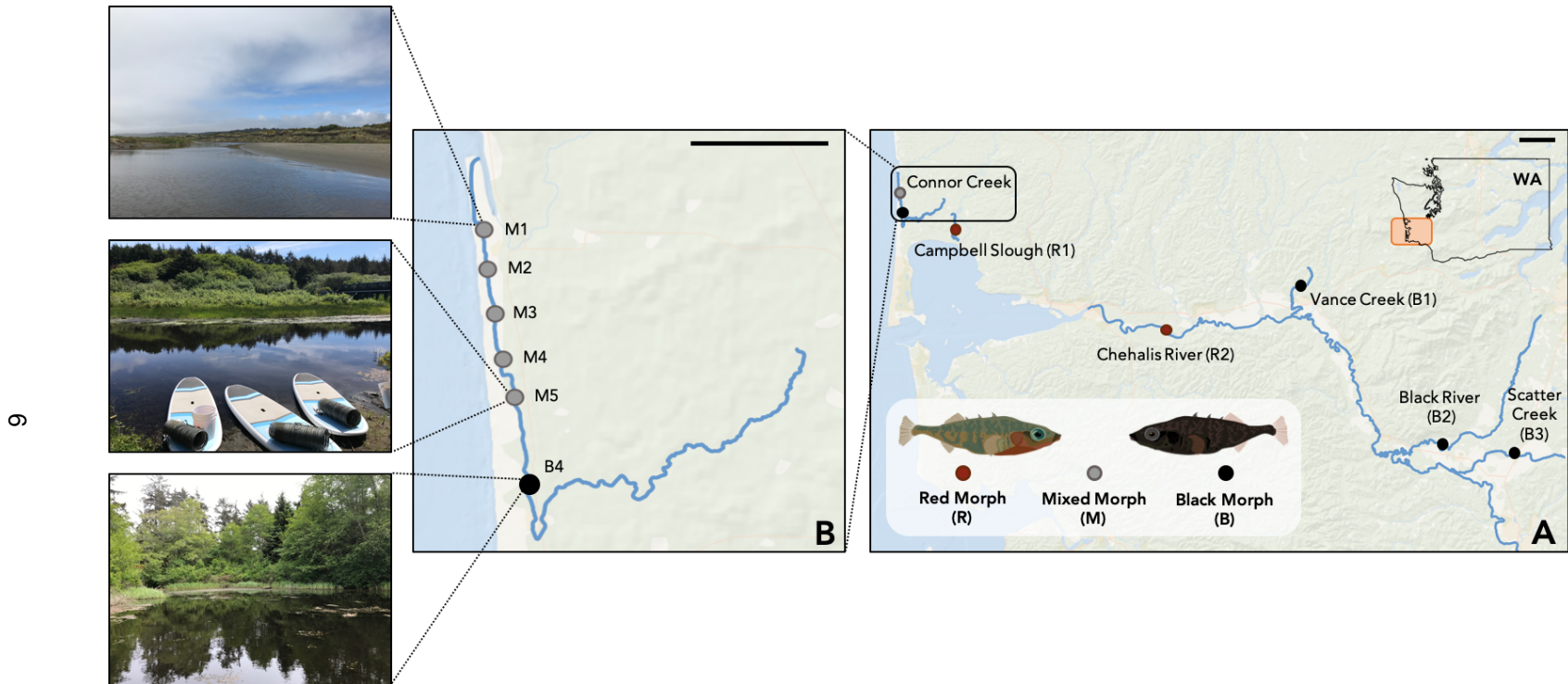


Figure 1.1. Washington sites used in morphological analyses (A). Connor Creek collection sites (B) mirror those of McPhail (1969). Sites where we collect red stickleback are denoted with “R”, sites where we collect black stickleback are denoted with “B”, and sites where we collect mixed stickleback are denoted with “M” for mixed Black bars in the top right corner of each panel correspond to a distance of five kilometers.

While we sampled five locations along the transect, fish did not appear to differ in morphology or color between locations. Thus, for the purpose of our phenotypic analyses, we hereafter refer to these five Connor Creek locations within our finer-scale approach as one collective “mixed” site, M1-M5. In the lab, fish were maintained in single sex 110-L holding tanks under controlled lighting and temperature conditions (17°C and a 12:12 light:dark schedule).

Colorimetric water collection

To confirm sensory drive as the prevailing theory behind the evolutionary switch from red nuptial coloration to black nuptial coloration (Reimchen 1989; Scott 2001; Boughman 2001) in our sampling sites, we collected three to five water samples from each site, as well as the five locations along the Connor Creek transect, for colorimetric analyses. We measured the transmittance of light through each water sample using a spectrophotometer at wavelengths of 340, 405, 490, 550, 595, and 650 nm, calibrating with distilled water (100% transmittance) before each new sample following Scott (2001).

Phenotypic data collection

We measured four morphological traits on males and females from 11 sites total (sample sizes are found in Table 1.1).

Table 1.1. Collection site details including site ID, color morph found at each, GPS coordinates, and the number of individuals phenotyped. Sites with only red fish are denoted with “R”, sites with only black fish are denoted with “B”, and sites with mixed fish are denoted with “M”.

Table 1.1.

Sites	Color Morph	GPS Coordinates	Shape & Size		Color	Plating
			Males	Females	Males	Males & Females
Campbell Slough (R1)	Red	47°2'40"N, 124°3'33" W	42	31	33	42
Chehalis River (R2)	Red	46°56'22"N, 123°18'46"W	57	49	21	41
Vance Creek (B1)	Black	46°59'48"N, 123°24'43"W	34	12	26	42
Black River (B2)	Black	46°49'45"N, 123°8'1"W	41	46	23	41
∞ Scatter Creek (B3)	Black	46°49'20"N, 123°3'11"W	40	22	22	42
Connor Creek (B4)	Black	47°4'11"N, 124°10'5"W	35	28	24	41
Connor Creek (M1)	Mixed	47°6'55"N, 124°10'52"W	5	-	5	30
Connor Creek (M2)	Mixed	47°6'26"N, 124°10'45"W	7	-	7	32
Connor Creek (M3)	Mixed	47°5'57"N, 124°10'39"W	6	-	6	26
Connor Creek (M4)	Mixed	47°5'29"N, 124°10'30"W	3	-	3	18
Connor Creek (M5)	Mixed	47°5'12"N, 124°10'20"W	2	-	2	46

To quantify shape, size, and color, we uniquely numbered and photographed live individuals using a digital camera (Canon PowerShot G15) under standardized lighting conditions inside of a photo box. The camera was placed at a fixed distance from a neutral background and we positioned fish on their right side below a millimeter ruler. All photographs were taken during the breeding season (June to September). When several photographs of the same fish existed, we used a random number generator to determine which image to analyze, ensuring that photographs of fish taken at particular time points in the breeding season were not selected preferentially. All shape, size, and color data were collected using the Image J package, FIJI (Schindelin et al. 2012). For each photograph, we set a scale factor using the ruler above the fish, cropped the image to only include the individual, and deleted the tailfin, as the tail does not always lay flat in photographs. This image was then used for the assessment of color, shape, and size.

Because female stickleback at sites containing both red and black male color morphs are drab, we only analyzed color in 172 males. All males expressed nuptial coloration at the time of photographing. We measured red and black coloration as a proportion of total body area from each image, after additionally removing the area of the eyeball from the image in ImageJ (Figure 1.2B). First, we selected red coloration using the Threshold Color plugin within FIJI ($Y=32$, $U=143$, $V=141$; following Wong et al. 2007), removing all pixels outside of these values (any area on the male that is not red). We converted the remaining selected pixels to 8-bit grayscale and calculated total red coloration by measuring the entire area of the pixels. To select black coloration, the image was converted to 8-bit grayscale, the threshold was set to 25, and the entire area of the pixels was measured. We determined total body area using the SIOX (Simple Interactive Object Extraction) segmentation tool to create a silhouette of the fish.

We carried out morphometric analyses to quantify shape of 462 male and female stickleback by placing 22 landmarks on each image and collecting their X-Y coordinates (Figure 1.2A). These landmarks have been previously established to best capture shape variation in stickleback (Walker and Bell 2000; Taylor et al. 2006; Albert et al. 2008; Cooper et al. 2011;

Malek et al. 2012; Head et al. 2013). We then quantified overall body size of males using standard length as our measure (Nagel and Schluter 1998; Head et al. 2009), by calculating the distance between landmarks one and 11 (the tip of the mouth to the base of the caudal fin). Our sampling regime did not include photos of females from the Connor Creek mixed sites (M1-M5) so they were excluded from shape and size analyses.

Following their natural death in the lab, we stored fish by collection site in jars containing 90% ethanol. To quantify lateral plating across morphs, we stained fish with Alizarin red following standardized methods in Cresko et al. (2004). We counted lateral body plates on both sides of 401 fish from 11 sites and additionally categorized each individual as having full, partial, intermediate, or low plating (Figure 1.2C).

Figure 1.2.

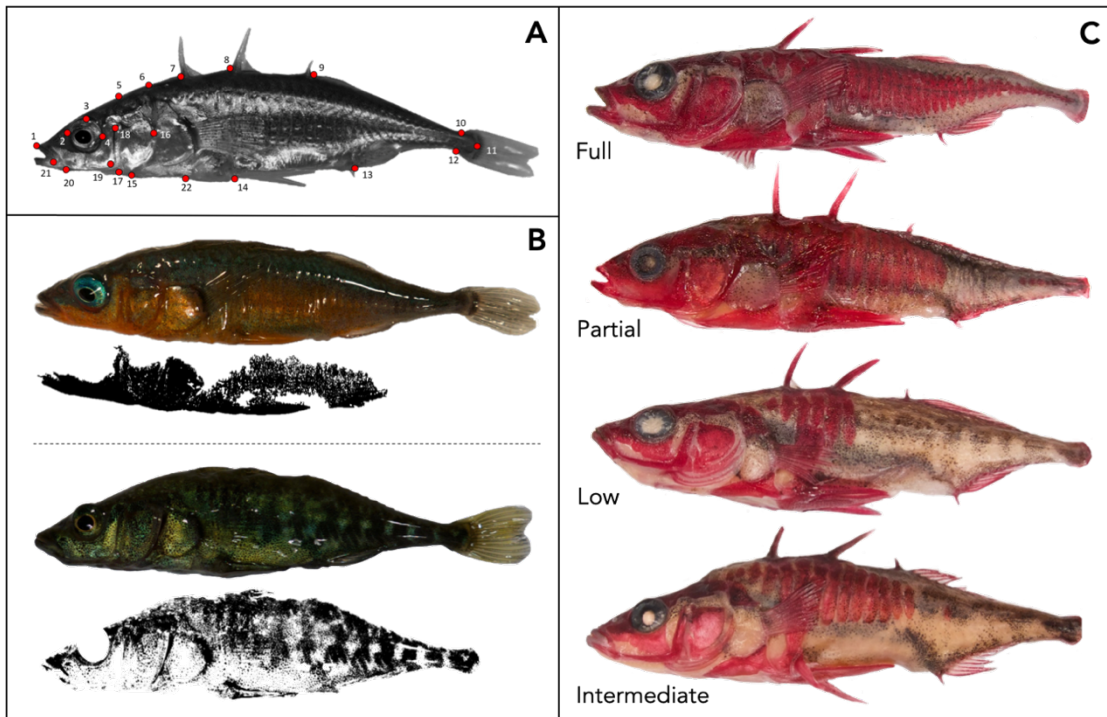


Figure 1.2. Color and morphological traits measured. A male displaying the 22 landmarks used to conduct shape and size analyses (A). A red male with quantification of red coloration (B; top) and a black male with quantification of black coloration (B; bottom). Four individuals representing each category of plating (C).

Following Bell (1982), we considered fish to be fully plated if they had a continuous row of plates from the head to the caudal peduncle, low plated if plating was strictly restricted to the abdominal region, and partially plated if they had both abdominal plating and a row of plating near the caudal peduncle that were separated by a gap with no plating. During the staining process, we discovered fish from our most recent collection in Connor Creek that could not fit into any of these categories. Similar to Bell et al. (2004), these atypical individuals were denoted as “intermediate”, as they had a row of plates that extended beyond the abdominal region but did not have a row of plating near the caudal peduncle (i.e. not low or partial plating).

Statistical analyses

Following Reimchen (1989) and Scott (2001), we used average transmittance at 405 nm as our standard measure of water color, as tannin staining is best indicated by low transmittance at shorter wavelengths and shorter wavelengths are the most variable among our collection sites. We conducted two one-way ANOVAs to compare the effect of sampling site on the transmittance of light through water samples. The first compared the average transmittance of light at 405 nm among all sites we categorize as red (R1 and R2), all sites we categorize as black (B1-B4), and the site we categorize as mixed (locations M1-M5). The second compared the average transmittance of light at 405 nm among sampling locations only within Connor Creek (B4 and M1-M5) to test for variation in water color within a single site.

To quantitatively determine if what we refer to as red and black morphs differ in coloration, we first performed a regression of black area on red area to obtain residuals for every individual, allowing us to represent color as a single variable. Increasingly negative residuals indicate redder fish whereas increasingly positive residuals indicate blacker fish. We then used one REML linear mixed model with residual color as the outcome variable, categorical color morph as a fixed effect, and site as a random effect to assess differences in male color among morphs, and another with site as a fixed effect to assess differences in male color among sites.

We conducted the analysis of morphometric data in MorphoJ version 2.0 (Klingenberg 2011). The landmark X-Y coordinates were imported into the program and, following Lackey and Boughman (2013), we used the Procrustes transformation to center, scale, and align the coordinates, allowing for comparisons of each landmark across images by controlling for the relative size and position of each individual. We used methods established by Drake and Klingenberg (2007), analyzing overall shape as a function of our continuous measure of color, to directly test for a relationship between shape and color, which we expect if body shape and color are correlated (Malek et al. 2012). We first performed a multivariate regression of the Procrustes transformed coordinates to calculate a shape score. We then used a mixed model with continuous (residual color) and categorical color (red, black, mixed) measures as fixed effects, site as a random effect, centroid size as a covariate, and the regression score representing shape as the outcome variable. The vectors of regression coefficients from these analyses can be thought of as shape changes per unit of color change. To determine how well each morph is classified by color and shape, we performed a linear discriminant analysis (LDA) in R using the packages “stats” (R Core Development Team 2010) and “MASS” (Venables and Ripley 2002) with categorical color as the grouping factor, and continuous color and the regression shape score as discriminators.

We performed a canonical variate analyses to visualize and statistically assess shape features that best distinguish groups from one another, comparing body shape between color morphs and sexes. We then used two principal component analyses (PCAs) to examine shape variation among males of different color morphs. The first PCA assessed variation in shape among males of all color morphs and from all sites. To more closely investigate the phenotypes of males from Connor Creek where color morphs are not as easily distinguished, the second PCA assessed variation in shape among only Connor Creek males (from sites B4 and M1-M5, but no red males). We then used PC1 and PC2 of the all-males PCA as outcome variables in linear models to test for variation in shape among categorical color morphs (mixed model, site = random effect) and among sites. We repeated this with the Connor Creek-male PCA, using PC1

and PC2 from this model as outcome variables and site as a fixed effect. To visualize the differences in shape among color morphs, we also performed a PCA for each categorical color morph separately and created wireframe graphs using the independent axes of body shape variation (PC1 and PC2) and compared them to the average shape of all males in MorphoJ.

To assess differences in size among male color morphs, we used a linear mixed model with length as the outcome variable, categorical color morph as a fixed effect, and site as a random effect. Finally, we used a linear mixed model with continuous plate count of both males and females as the outcome variable, categorical color morph as a fixed effect, and site as a random effect to assess differences in lateral plate count among color morphs.

Results

Water color

The transmittance of light through water samples at 405 nm varied across sites that we categorized as red, black, or mixed ($F_{2,47} = 3.87$, $p = 0.028$; Figure 1.3A). Red sites have higher transmittance than black sites (Tukey's HSD: estimate \pm SE; 1.65 ± 0.018 , $p = <0.05$), but transmittance in the mixed site (locations M1-M5) did not differ from red sites (1.14 ± 0.50 , $p = >0.05$) or from black sites (0.51 ± 0.65 , $p = >0.05$). Transmittance also varied within Connor Creek ($F_{5,24} = 49.24$, $p = <0.0001$; Figure 1.3B).

Figure 1.3.

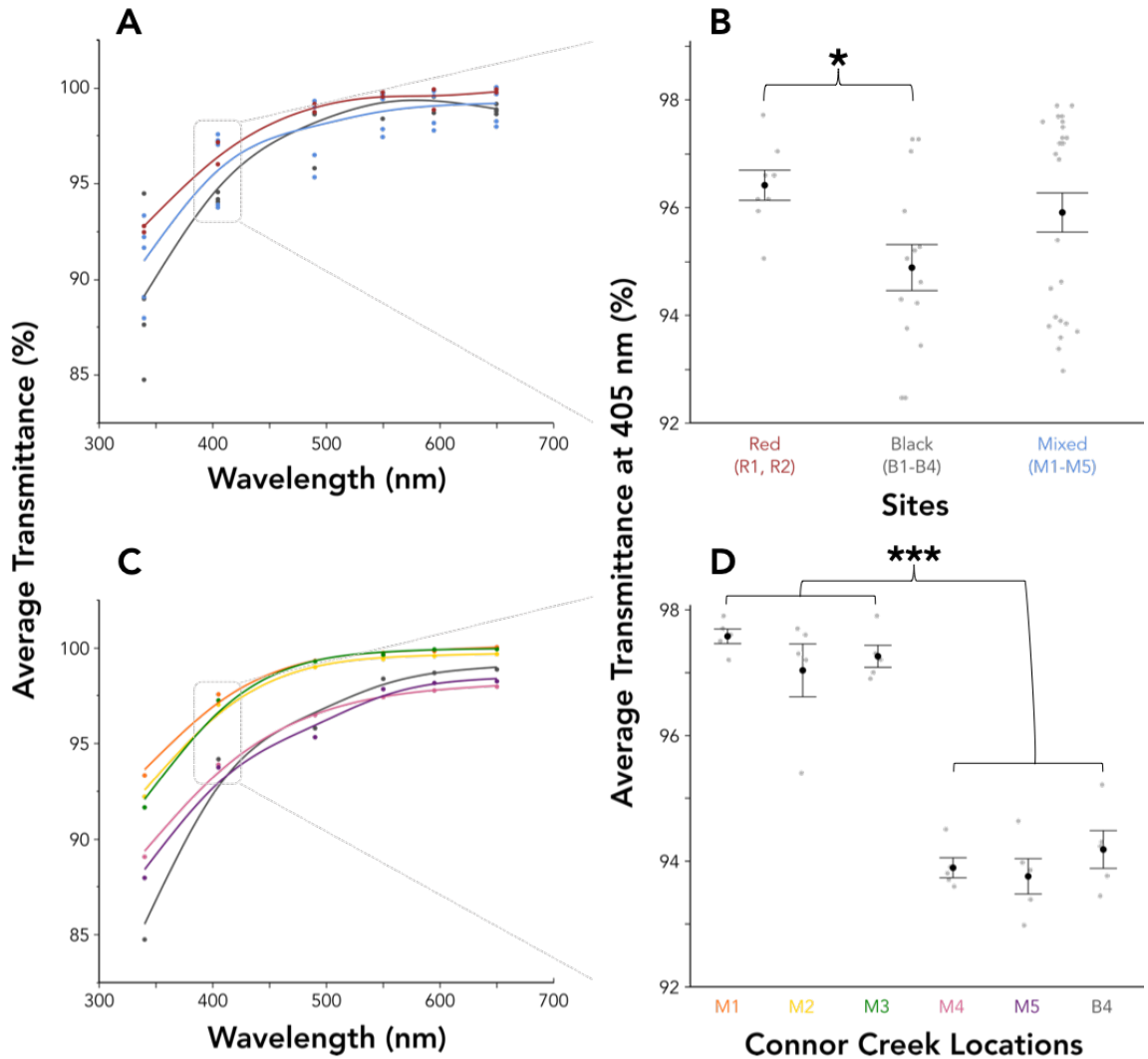


Figure 1.3. Average transmittance of light through water samples from all collection sites (A) and from the six collection locations only within Connor Creek (C) at a range of wavelengths. Each data point represents the mean of three to five replicate samples. At 405 nm, transmittance of light differed between red and black sites, but not between red and mixed or black and mixed sites (B). Within Connor Creek, transmittance at 405 nm did not differ among M1, M2, and M3 nor did it differ among M4, M5, and B4, but transmittance differed in all comparisons within these two groupings (D). Grey points represent raw values, black points and bars represent the least square means from the analysis plus or minus standard error, and asterisks indicate significant differences using Tukey's HSD (* $p < 0.05$, *** $p < 0.0001$).

The transmittance of light did not differ among M1, M2, and M3, but each of the three locations had higher transmittance than M4, M5, and B4. The transmittance of light did not differ among M4, M5, and B4 (Tukey's HSD pairwise comparisons in Table 1.2).

Table 1.2. Tukey's HSD pairwise comparisons for transmittance of light at 405 nm among Connor Creek sampling locations. Highlighted cells represent significantly different pairwise comparisons (alpha = 0.05, *p = <0.0001).

Table 1.2.

	M2	M3	M4	M5	B4
M1	0.54 ± 0.37	0.32 ± 0.37	*3.68 ± 0.37	*3.82 ± 0.37	*3.39 ± 0.37
M2	-	0.22 ± 0.37	*3.14 ± 0.37	*3.28 ± 0.37	*2.85 ± 0.37
M3	-	-	*3.36 ± 0.37	*3.50 ± 0.37	*3.07 ± 0.37
M4	-	-	-	0.14 ± 0.37	0.29 ± 0.37
M5	-	-	-	-	0.43 ± 0.37

Figure 1.4.

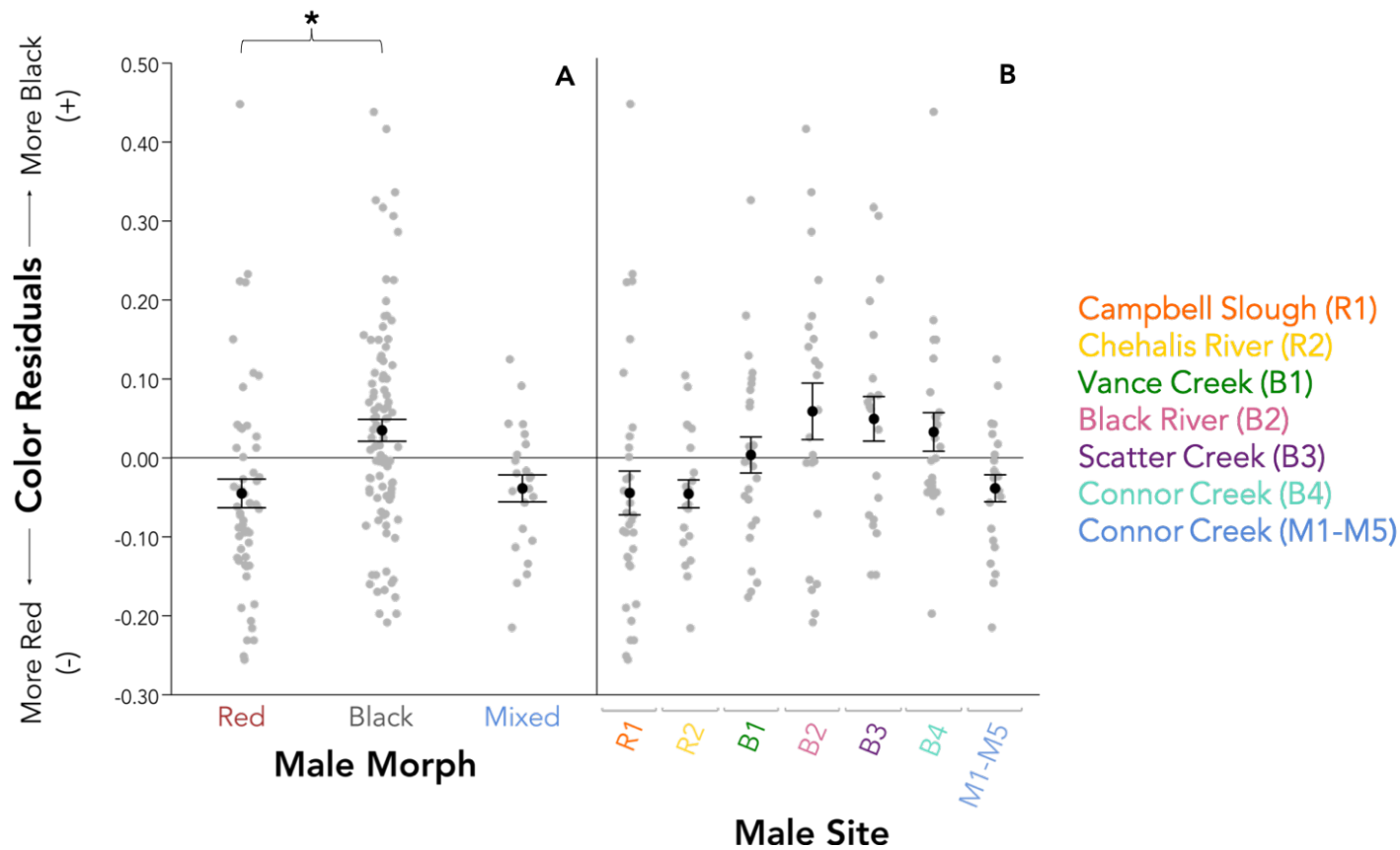


Figure 1.4. The residual color scores of males differed among morphs (left) and also among sampling sites (right). Red and black males differ in color, but mixed males do not differ from either (A). Sites within a morph do not significantly differ in color (B). Grey points represent raw values, black points and bars represent the least square means from the analysis plus or minus standard error, and asterisks indicate significant differences using Tukey's HSD ($p < 0.05$).

This strongly suggests a cline in transmittance, wherein water is less tannin stained as the creek approaches the ocean.

Color

We found quantitative differences in the color of male stickleback commonly called red and black ($F_{2,3.51} = 10.96$, $p = 0.031$; Figure 1.4A). Males commonly categorized by researchers (by eye) as “red” were significantly redder than those commonly categorized as “black” (Tukey’s HSD, $\alpha = 0.05$: -0.079 ± 0.018 , $p = <0.05$), and black male stickleback were significantly blacker than red stickleback. Consistent with our anecdotal observations, males from the five locations along Connor Creek where we could not definitively categorize them as red or black (collectively referred to as “mixed”), were intermediate in quantitative color and did not differ from either red (0.00063 ± 0.027 , $p = >0.05$) or black fish (-0.073 ± 0.025 , $p = >0.05$). We also found overall differences in male color by site ($F_{6,165} = 3.00$, $p = 0.0083$; Figure 1.4B), but there were no significant pairwise differences among sites using Tukey’s HSD.

Color and shape

Overall, we found that color and shape are correlated in this system. There was a significant relationship between continuous color variation and shape variation in male morphs ($\chi^2_1 = 13.72$, $p = 0.0002$) that is dependent on categorical color ($\chi^2_2 = 23.73$, $p = <0.0001$; Figure 1.5).

Figure 1.5.

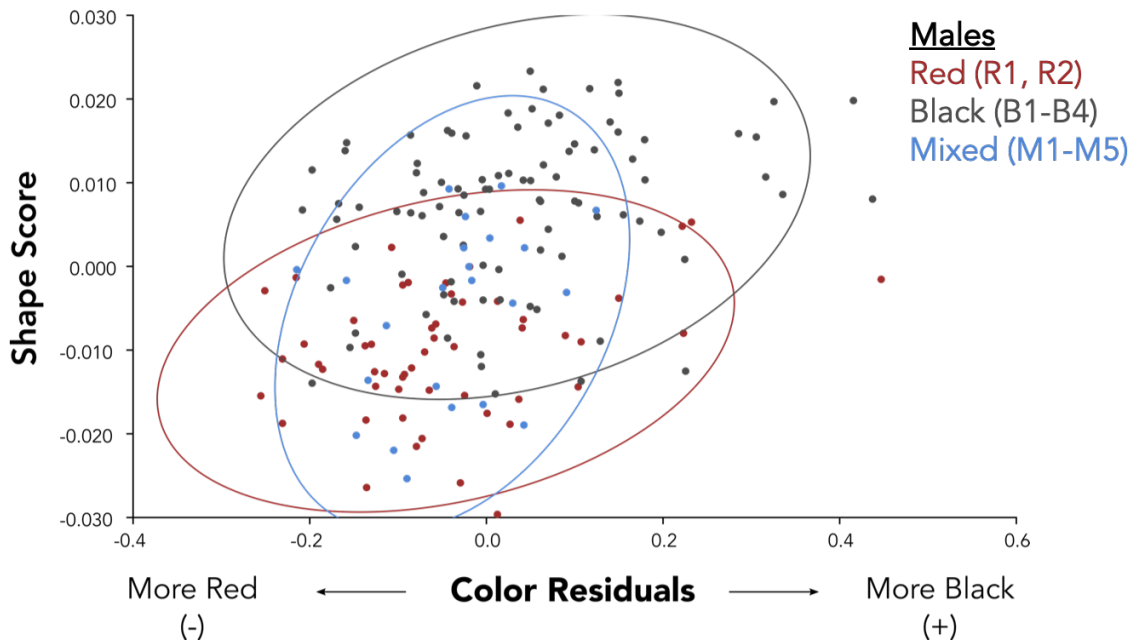


Figure 1.5. Male residual color and body shape are correlated, but the relationship between residual color and shape scores of males differs among morphs. The relationship between color and shape differs for red and black males, and for black and mixed males, but it does not differ for red males and mixed males. Ellipses represent 95% confidence intervals.

The relationship between color and shape differs between red and black males (Tukey's HSD, $\alpha = 0.05$: -0.016 ± 0.0016 , $p = <0.001$), and between black and mixed males (-0.011 ± 0.0024 , $p = <0.001$), but the relationship between color and shape did not differ between red and mixed males (-0.0045 ± 0.0024 , $p = 0.13$). The LDA showed that 99.83% of the discriminability is explained by LD1 (Figure 1.6).

Figure 1.6.

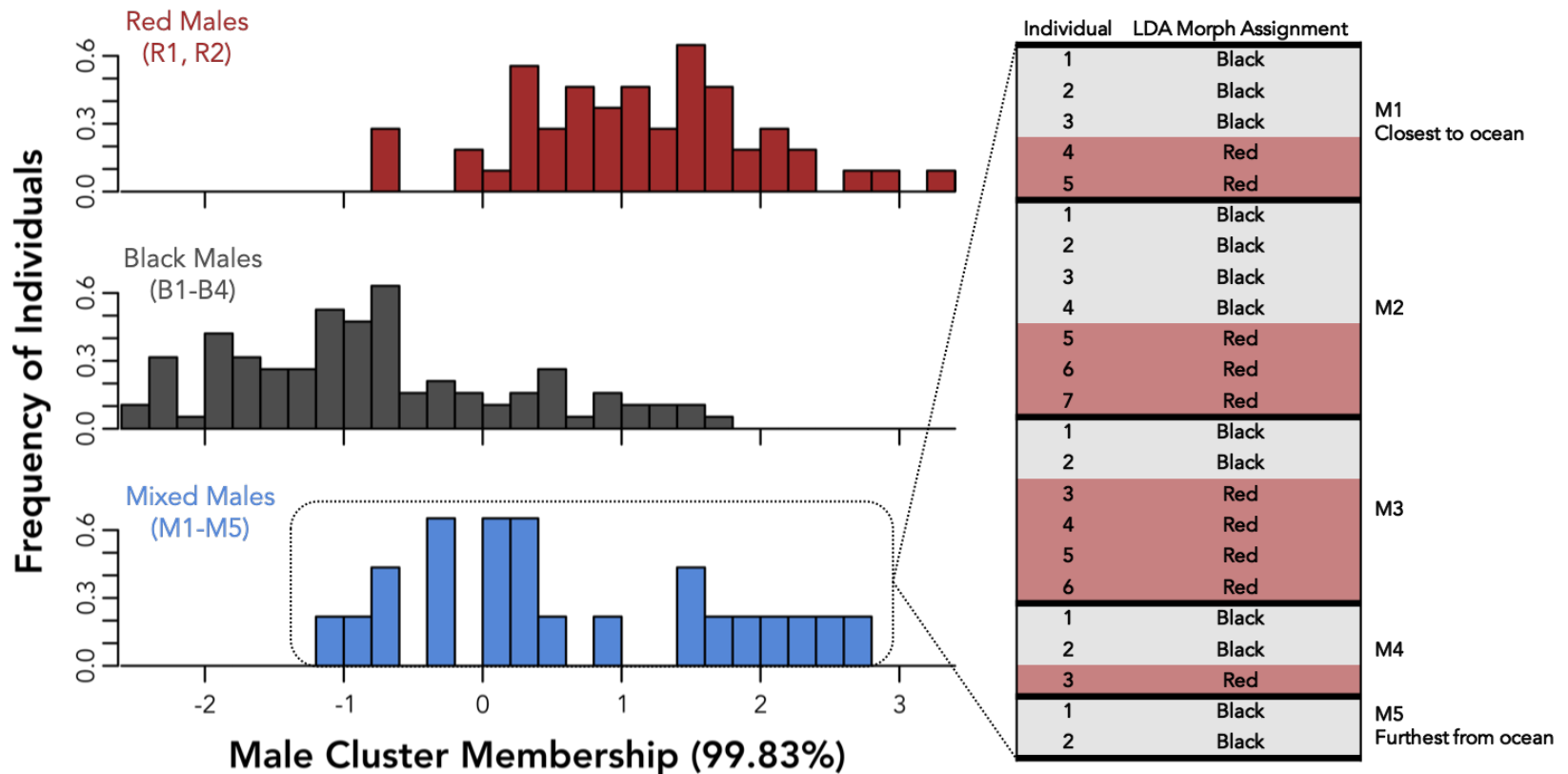


Figure 1.6. Linear discriminant analysis of males classified by shape and color. Together, male color and shape correctly classified most individuals from allopatric red sites as red, and most individuals from allopatric black sites as black. Nearly half of the individuals from the Connor Creek mixed site (locations M1-M5) were classified as red and half were classified as black (see Table 2 for LDA assignments and proportions).

Of the individuals we categorize as red (sites R1 and R2), 74.1% were classified as red and 25.9% were classified as black by the LDA. Of the individuals we categorize as black (sites B1-B4), 85.3% were classified as black and 14.7% were classified as red by the LDA. No individuals of any morph were classified as mixed by the LDA, however, of the individuals we categorize as mixed (site M1-M5), 43.5% were classified as red and 56.5% were classified as black (Table 1.3).

Table 1.3. Proportion of stickleback assigned to red, black, or mixed color morphs based on a linear discriminant analysis using color and shape.

Table 1.3.

	LDA Classification		
	Red	Black	Mixed
Red (N=54)	74.1%	25.9%	0%
Black (N=95)	14.7%	85.3%	0%
Mixed (N=23)	43.5%	56.5%	0%

Shape

When fish were placed into five groups by sex and morph (red females, black females, red males, black males, and mixed males), we found significant variation in overall body shape between all groups (Figure 1.7; Table 1.4).

Figure 1.7.

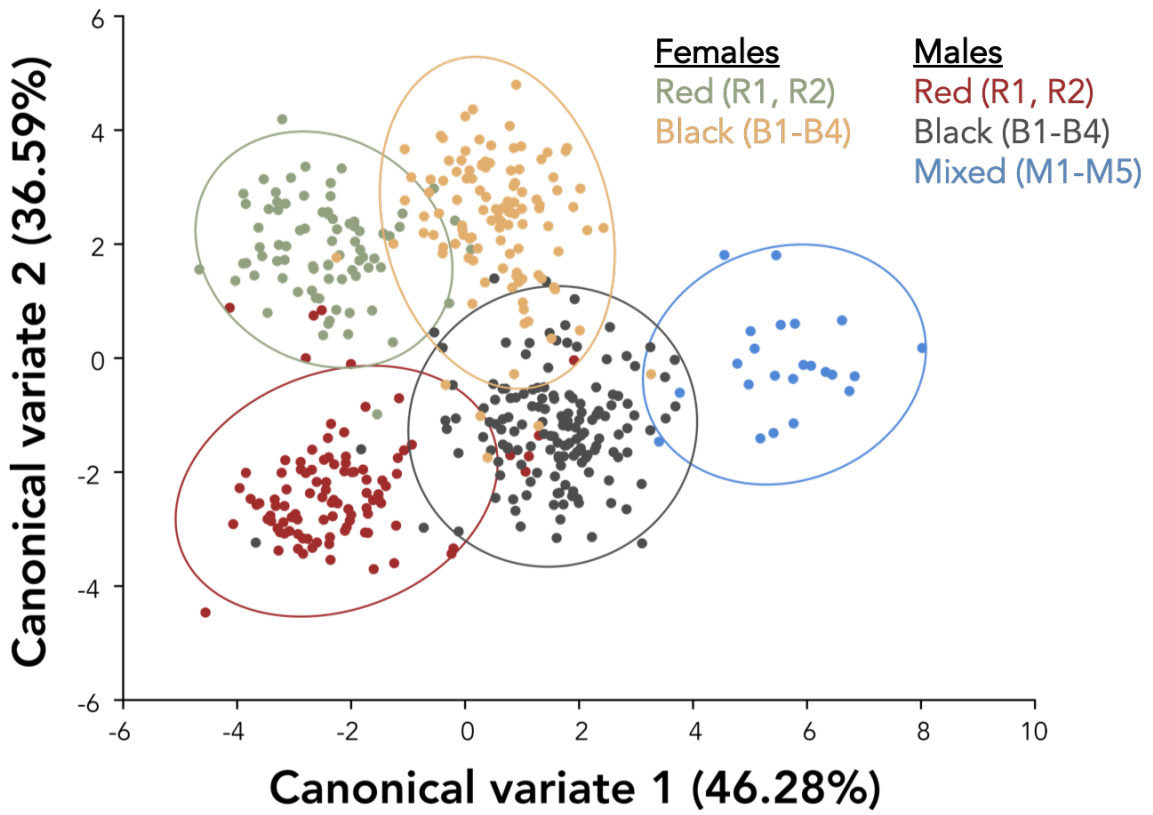


Figure 1.7. Variation in shape between sexes and color morphs. There was significant variation in shape in all pairwise comparisons of the five groups (see Table 3). Ellipses represent 95% confidence intervals.

Table 1.4. Procrustes distances and p-values comparing shape across sex and color morph.

Table 1.4.

	Black Females		Red Males		Black Males		Mixed Males	
	Procrustes Distance	p-value	Procrustes Distance	p-value	Procrustes Distance	p-value	Procrustes Distance	p-value
Red Females	0.028	<0.0001	0.053	<0.0001	0.050	<0.0001	0.065	<0.0001
Black Females	-	-	0.056	<0.0001	0.041	<0.0001	0.054	<0.0001
Red Males	-	-	-	-	0.030	<0.0001	0.052	<0.0001
Black Males	-	-	-	-	-	-	0.036	<0.0001

Within a color morph, shape significantly differed between the sexes (Procrustes distance $_{\text{red female}} - \text{red male} = 0.053$, $p = <0.0001$; Procrustes distance $_{\text{black female}} - \text{black male} = 0.041$, $p = <0.0001$), and within a sex, shape significantly differed between the morphs (Procrustes distance $_{\text{red female}} - \text{black female} = 0.028$, $p = <0.0001$; Procrustes distance $_{\text{red male}} - \text{black male} = 0.030$, $p = <0.0001$; Procrustes distance $_{\text{red male}} - \text{mixed male} = 0.052$, $p = <0.0001$; Procrustes distance $_{\text{black male}} - \text{mixed male} = 0.036$, $p = <0.0001$). CV1 explained 46.28% of the total variation in shape and CV2 explained 36.59% of the total variation in shape.

In our first principal component analysis investigating shape differences among all males, the major axis of phenotypic variation, PC1, explained 37.48% of the total variation in shape and the second axis of phenotypic variation, PC2, explained 14.20% of the total variation in shape (Figure 1.8).

Figure 1.8.

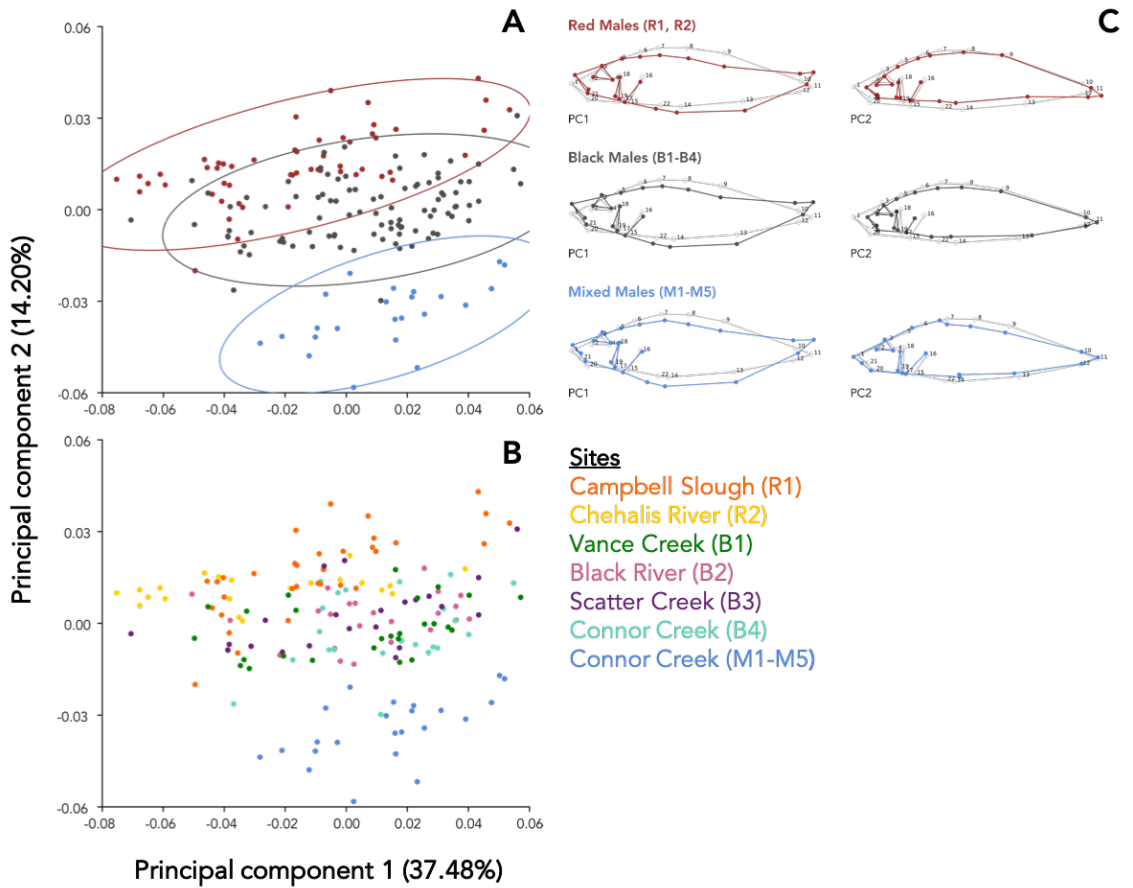


Figure 1.8. Principal component analysis of shape among all males by color morph (A) and site (B). PC1 and PC2 scores differed among morphs and sites (see Tables 4-7 for all Tukey's HSD pairwise comparisons). Ellipses represent 95% confidence intervals. Wireframes showing differences in shape between color morphs (C). In each case, the underlying grey wireframe corresponds to the average of the entire male dataset, and the overlaying colored wireframes show how the shape of males of each color morph differ from the average on PC1 (left) and PC2 (right).

The linear model confirmed PC1 ($F_{2,4.12} = 8.72$, $p = 0.033$) and PC2 ($F_{2,4.00} = 82.89$, $p = 0.0006$) scores differed among red, black, and mixed male color morphs (Figure 1.8A; Tukey's HSD pairwise comparisons in Tables 1.5 and 1.6).

Table 1.5. Tukey's HSD pairwise comparisons for PC1 scores of male shape among color morphs. Highlighted cells represent significantly different pairwise comparisons ($\alpha = 0.05$).

Table 1.5.

	Black Males	Mixed Males
Red Males	0.023 ± 0.00063	0.030 ± 0.0090
Black Males	-	0.0071 ± 0.00083

Table 1.6. Tukey's HSD pairwise comparisons for PC2 scores of male shape among color morphs. Highlighted cells represent significantly different pairwise comparisons ($\alpha = 0.05$).

Table 1.6.

	Black Males	Mixed Males
Red Males	0.015 ± 0.0027	0.049 ± 0.0038
Black Males	-	0.034 ± 0.0035

PC1 ($F_{6,165} = 5.88$, $p = <0.0001$) and PC2 ($F_{6,165} = 60.19$, $p = <0.0001$) scores also differed among sites (Figure 1.8B; Tukey's HSD pairwise comparisons in Tables 1.7 and 1.8).

Table 1.7. Tukey's HSD pairwise comparisons for PC1 scores of male shape among sites. Highlighted cells represent significantly different pairwise comparisons (alpha = 0.05).

Table 1.7.

	R2	B1	B2	B3	B4	M1-M5
R1	0.018 ± 0.0077	0.013 ± 0.0073	0.017 ± 0.0075	0.012 ± 0.0076	0.020 ± 0.0074	0.023 ± 0.0075
R2	-	0.031 ± 0.0081	0.035 ± 0.0084	0.030 ± 0.0085	0.038 ± 0.0083	0.041 ± 0.0084
B1	-	-	0.0037 ± 0.0079	0.0017 ± 0.0080	0.013 ± 0.0073	0.0092 ± 0.0079
B2	-	-	-	0.0054 ± 0.0083	0.0028 ± 0.0081	0.0056 ± 0.0082
B3	-	-	-	-	0.0082 ± 0.0082	0.011 ± 0.0083
B4	-	-	-	-	-	0.0028 ± 0.0081

Table 1.8. Tukey's HSD pairwise comparisons for PC2 scores of male shape among sites. Highlighted cells represent significantly different pairwise comparisons (alpha = 0.05).

Table 1.8.

	R2	B1	B2	B3	B4	M1-M5
R1	0.0058 ± 0.0029	0.019 ± 0.0028	0.015 ± 0.0029	0.014 ± 0.0029	0.020 ± 0.0028	0.052 ± 0.0029
R2	-	0.013 ± 0.0031	0.0095 ± 0.0032	0.0086 ± 0.0032	0.014 ± 0.0032	0.046 ± 0.0032
B1	-	-	0.0035 ± 0.0030	0.0044 ± 0.0031	0.0011 ± 0.0030	0.033 ± 0.0030
B2	-	-	-	0.0009 ± 0.0031	0.0046 ± 0.0031	0.036 ± 0.0031
B3	-	-	-	-	0.0055 ± 0.0031	0.037 ± 0.0031
B4	-	-	-	-	-	0.032 ± 0.0031

The wireframe graphic of PC1 depicts how each morph deviates in body shape from the average of the entire male dataset whereas PC2 depicts how each morph deviates in face shape from the average of the entire male dataset (Figure 1.8C).

In our second principal component analysis among only males from Connor Creek, PC1 explained 29.43% of the total variation in shape and PC2 explained 19.89% of the total variation in shape. Together the two axes of variation largely differentiate black from mixed males within Connor Creek (Figure 1.9).

Figure 1.9.

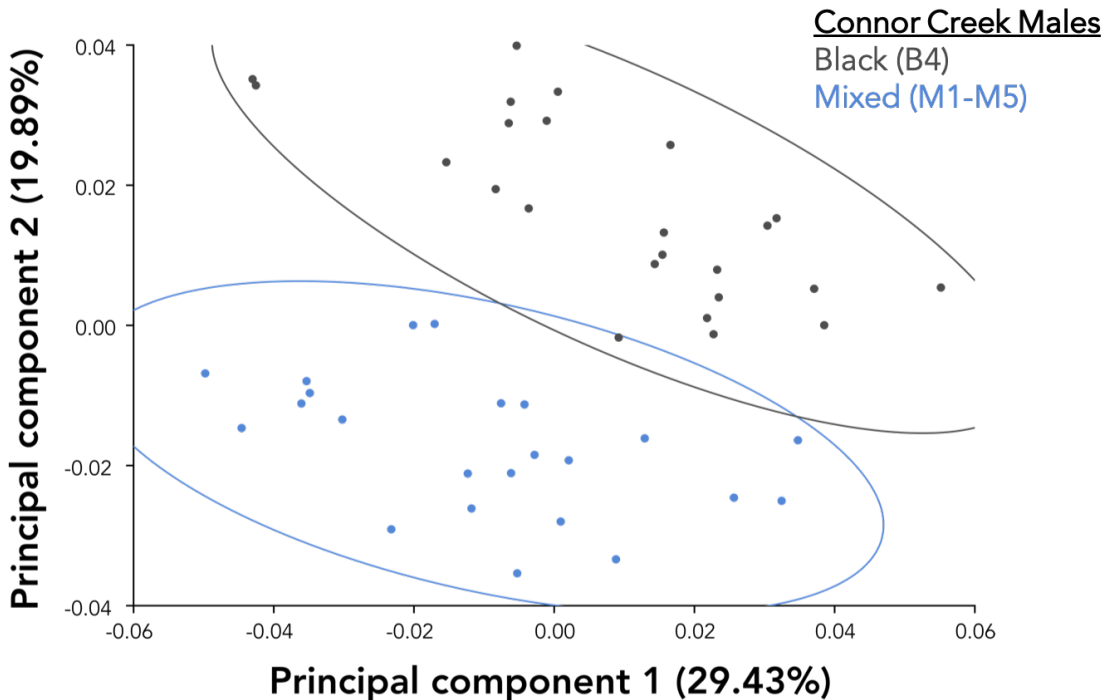


Figure 1.9. Principal component analysis of male shape among only Connor Creek morphs. The color morphs differ in both PC1 and PC2. Ellipses represent 95% confidence intervals.

The linear model confirmed that there was a significant difference in PC1 ($F_{1,46} = 7.75$, $p = 0.008$) and PC2 ($F_{1,46} = 102.08$, $p < 0.0001$) scores between the two color morphs.

Size

Overall, we found differences in the size of male stickleback of different color morphs ($F_{2,6.51} = 29.49$, $p = 0.0005$; Figure 1.10).

Figure 1.10.

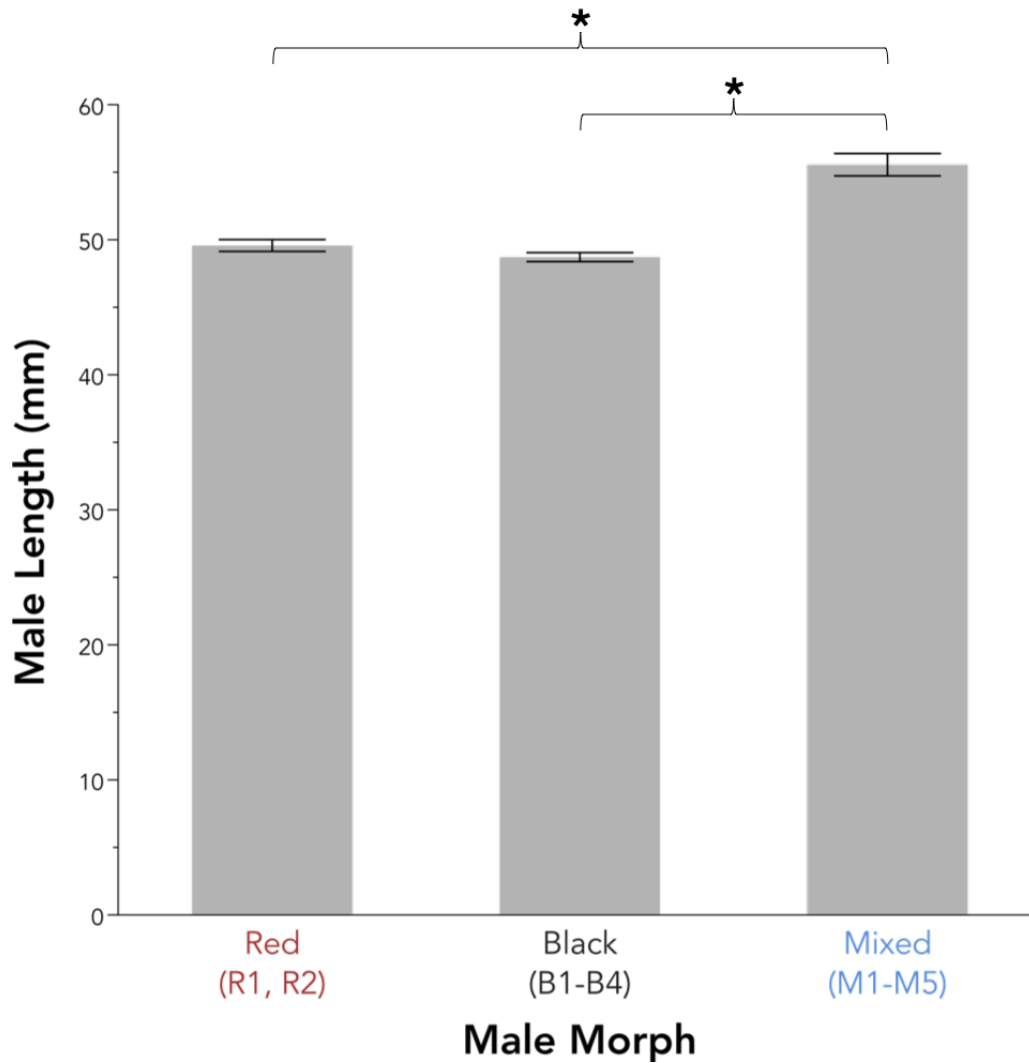


Figure 1.10. Mixed males are larger than red males and larger than black males, but red and black males do not differ in size. Bars represent the least square means from the analysis plus or minus standard error and asterisks indicate significant differences using Tukey's HSD ($p < 0.05$).

Red males were 49.59 ± 0.38 mm in length. Black males were 48.71 ± 0.32 mm in length. And, mixed males were 55.56 ± 0.83 mm in length. Red males and black males do not differ in size (Tukey's HSD, alpha = 0.05: 0.89 ± 0.49 , $p = >0.05$), but mixed males are significantly larger than both red (5.96 ± 0.91 , $p = <0.05$) and black males (6.85 ± 0.88 , $p = <0.05$).

Plating

We also found differences in lateral body plating among color morphs ($F_{2,7.47} = 310.12$, $p = < 0.0001$; Figure 1.11).

Figure 1.11.

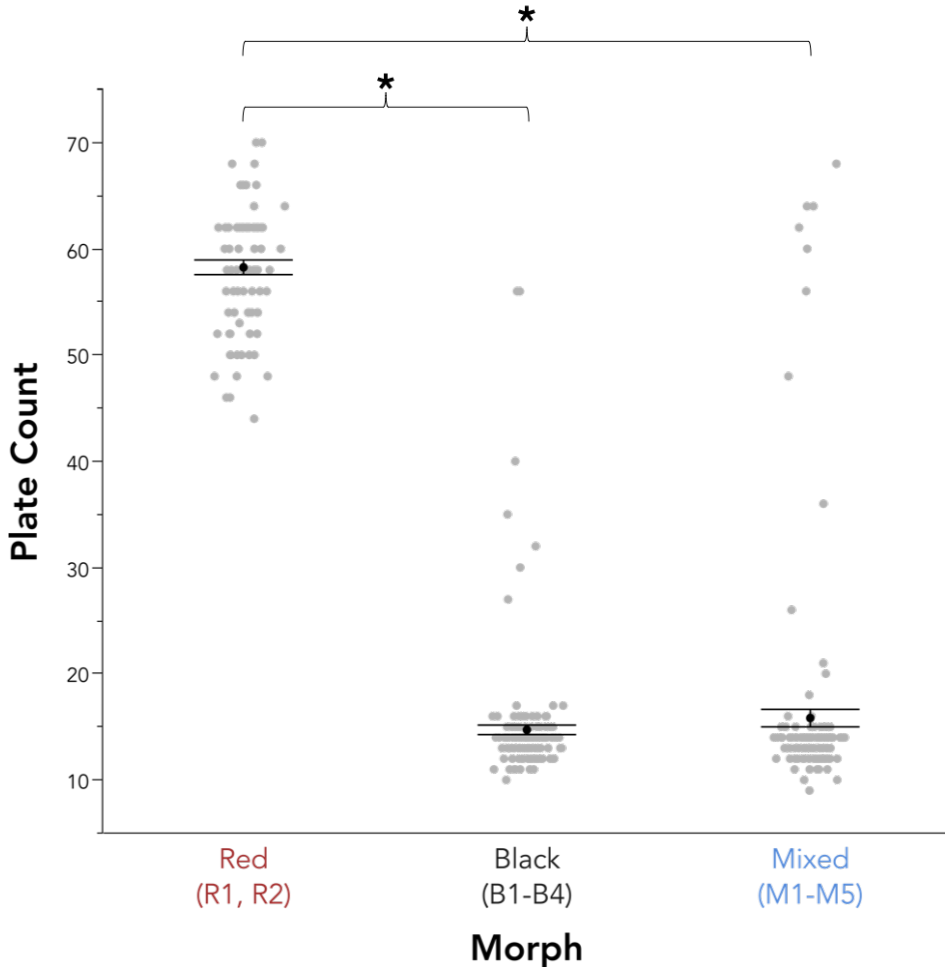


Figure 1.11. Lateral plate count of both males and females among color morphs. Black and mixed fish do not differ in lateral plate count, but red fish have significantly more body plating than both black fish and more body plating than mixed fish. The grey points represent raw values, the black points and bars represent the least square means from the analysis plus or minus standard error, and asterisks indicate significant differences using Tukey's HSD ($p < 0.05$).

98.8% of red fish were fully-plated and only 1.2% of individuals were partially-plated. 95.8% of black fish were low-plated, 3.0% were partially-plated, and 1.2% were fully plated. 92.1% of mixed fish were low-plated, 0.7% were partially-plated, 2.6% were intermediately-plated, and 4.6% were fully-plated (Table 1.9).

Table 1.9. Number of individuals in each color morph categorized by plate morph.

Table 1.9.

Color Morph	Plate Morph			
	Low	Partial	Intermediate	Full
Red	0	1	0	82
Black	159	5	0	2
Mixed	140	1	4	7

All four plate morphs were observed among individuals of the mixed color morph, and the atypical, intermediate plate morph – an uncommon and rare occurrence (Bell 2004) – was only observed among individuals of the mixed color morph. On average, red males had a plate count of 57.61 ± 1.52 plates, black males had a plate count of 14.70 ± 1.07 plate, and mixed males had a plate count of 15.61 ± 1.12 plates. Black and mixed fish did not differ in lateral plate count (Tukey's HSD, $\alpha = 0.05$: 0.90 ± 1.55 , $p = >0.05$), but red fish have significantly more body plating than both black (42.91 ± 1.86 , $p = <0.05$) and mixed fish (42.00 ± 1.89 , $p = <0.05$).

Discussion

Among freshwater sites in southwest Washington, we found clear phenotypic divergence between red and black stickleback morphs in color, shape, and plating, and further support for sensory drive as the prevailing mechanism behind the rapid, evolutionary switch in nuptial coloration in this system. We found a region in Connor Creek with a cline in water color (transmittance at 405 nm) and associated habitat where the “mixed” morph has traits that are sometimes intermediate to the red and black morphs and sometimes divergent (e.g. size). In Connor Creek, habitat variation may play a role in the maintenance of multiple morphs in very close proximity and perhaps contributes to hybridization.

Divergence in color and support for sensory drive

Red coloration in the threespine stickleback is a well-established component of sexual signaling (Semler 1971; Milinski and Bakker 1990; McKinnon 1995) and black coloration, which is less well-studied, has been documented in at least three different geographic regions – southwest Washington, the Queen Charlotte Islands, and British Columbia (McPhail 1969; Semler 1971; Reimchen 1989; Boughman 2001). When we assessed color as a continuous variable (the residuals of black coloration onto red coloration), we confirmed that red and black stickleback are different from one another, and that the mixed morph is intermediate in color to red and black (Figure 1.4A). Sites within a morph do not vary significantly in color (Figure 1.4B). In several of these cases, differences in the light environment have been implicated in the switch from red to black male coloration. When there are high concentrations of dissolved organic compounds, such as tannins, in freshwater environments, short wavelength light is filtered out of the visible spectrum, producing a background of red-shifted light (Reimchen 1989). The black male sexual signal is highly contrasted against these tannin-stained habitats whereas the red male sexual signal is highly contrasted against the green-shifted light of most clear, unstained freshwater habitats (Reimchen 1989; Scott 2001; Boughman 2001). Thus, if sensory drive plays a role in this shift in color, we would expect the distribution of color morphs to align with the transmission properties of their environments. Indeed, we found that the transmittance of light through water at 405 nm was higher in sites where we collected only red stickleback and lower in sites where we collected only black stickleback, indicating that the black morph is distributed in environments with high tannin staining (Figure 1.3A).

Interestingly, the average transmittance at the short wavelength end of the visible spectrum (405 nm) of the mixed site falls intermediate to that of the red and black sites (Figure 1.3B), which is in accord with the intermediate coloration of the mixed morph relative to the red and black morphs. When we assessed each location within Connor Creek separately, we found evidence of an environmental gradient over this short distance; transmittance of light through water at 405 nm is higher at the three locations closest to the ocean than the three locations

furthest inland (Figure 1.3D; Table 1.2). In addition to a transition in transmission properties, we observed that the surrounding environments drastically changed from areas with high vegetation and deep water (B4) to sand dunes and shallow water (M1) as the creek approached the Pacific Ocean (Figure 1.1). Given that our water transmittance data support sensory drive, we expected that stickleback would be more phenotypically similar to the red morph in the location closest to the ocean (M1), where water is relatively clear and unstained, and gradually shift to an appearance more phenotypically similar to the black morph in the location furthest inland (B4), where water is red-shifted and tannin-rich. Instead of a phenotypic cline, we found that nearly half of the males we categorized as belonging to the mixed morph were classified as red and half were classified as black by the LDA, and that they were distributed almost evenly throughout the first four mixed locations (i.e. red-like fish were not only found in locations closer to the ocean and black-like fish were not only found in locations further inland; Figure 1.6; Table 1.3). It is possible that the lack of a phenotypic cline is due to the migration patterns of stickleback and the dramatic habitat variation we observe in Connor Creek. Given that freshwater stickleback can travel up to five kilometers to breeding sites and anadromous migrants can travel at least 10 kilometers to freshwater breeding sites (Snyder and Dingle 1989), individuals may be freely interbreeding along the creek, preventing the establishment of a measurable gradient across a short geographical range.

Divergence in shape and size

Body shape and size are well-studied components of sexual signaling in the marine-benthic and benthic-limnetic stickleback species complexes, and have been shown to vary both between sexes (Cooper et al. 2011) and between morphs in several populations (Taylor et al. 2006; Albert et al. 2008; Malek et al. 2012; Head et al. 2013). However, little is known about how shape and size diverge between morphs that inhabit different freshwater river or stream habitats. Here, we found variation in shape of stickleback from streams in southwest Washington between sexes and morphs (Figure 1.7; Table 1.4). Among only males, body shape differs between red and black morphs and sites (Figures 1.8A and 1.8B; Tables 1.5 and 1.6), but size does not

(Figure 1.10). Mixed males differed in shape from both red and black fish on one axis of a PCA (PC2; Figure 1.8A, Table 1.6) and by site (Figure 1.8B; Table 1.8). As depicted in the wireframe graphics, PC1 appears to best explain variation in body depth and shape, whereas PC2 appears to best explain variation in face structure and shape (Figure 1.8C). Thus, mixed males differ from red and black males primarily in face shape. Additionally, mixed males were also larger than red and black males (Figure 1.10). Among only males within Connor Creek, shape differed between the mixed morph and the upstream black morph (Figure 1.9). In the benthic-limnetic pair, differences in body shape arose by adaptation to local foraging and predator environments (reviewed in McKinnon and Rundle 2002). In our red-black species pair, it is possible that divergent natural selection has first led to divergence in shape from anadromous ancestors by adapting to freshwater environments (McPhail 1994), which may be followed by divergence in shape of the morphs through adaptation to specialized and different niches.

Alternatively, animals often examine more than one signal simultaneously when assessing competitors (Candolin and Voight 2001) and potential mates (Candolin 2003). Evolutionary changes in one sexually selected trait may be correlated with changes in others and are simultaneously under sexual selection in this system. When traits are correlated, through pleiotropy or linkage disequilibrium, direct selection on one may consequently lead to indirect selection on an associated trait (Brooks and Endler 2001). Malek et al. (2012) found that markers associated with male color were significantly associated with body shape in a quantitative trait locus analysis of benthic and limnetic stickleback, motivating our assessment of relationships between color and shape in SW Washington stickleback. We found that residual color and shape are indeed correlated, and that this relationship differs among color morphs (Figure 1.5). The relationship between color and shape differs for red and black males, and for black and mixed males, but it does not differ for red and mixed males. In addition to expressing preferences for extensive red coloration, there is also evidence that female stickleback have preferences for male body shape in some groups (Head et al. 2013). Male color and shape may thus be correlated through simultaneous direct natural selection on both traits during adaptation to freshwater

environments, because of sexual selection driven by female preference for both traits, or through indirect selection of one as a byproduct of direct selection on the other. Ultimately, the relationship between male color and shape suggests that when one is favored by natural or sexual selection, we might expect the other to evolve in concert.

Variation and surprises in lateral body plating

While the overwhelming majority of fish from red sites were almost entirely fully-plated, black and mixed fish were predominately low-plated with few partial, intermediate, and full morphs (Table 1.9; Figure 1.11). The occurrence of fully-plated individuals in red sites is unusual, in that we expect a loss or reduction in body armor following invasions from oceanic to freshwater environments (Hagen and Gilbertson 1973; Bell and Foster 1994). However, fully-plated populations have been documented in this region before (Hagen and Gilbertson 1973). The presence of fully-plated stickleback in Washington rivers could indicate that natural selection has favored the maintenance or reappearance of extra lateral plates in certain habitats. Alternatively, fully-plated red fish may live in environments subject to more or different predators than low-plated black fish or could be recently introduced, marine stickleback either through the migration of anadromous populations or through anthropogenic transfer from coastal to freshwater sites.

Though the number of plates did not differ between mixed and black morphs, it is interesting to note the unexpected presence of the intermediately-plated individuals within the mixed morph, which to our knowledge, has not before been documented in this region. In Loburg Lake, Alaska, Bell et al. (2004) also discovered rare intermediately-plated individuals and suggested that this plate morph does not occur in older polymorphic populations and is likely the result of novel allele combinations generated during adaptive radiation.

Accumulation of evidence for Connor Creek as a potential hybrid zone

We have established that a suite of traits differs between the red and black stickleback morphs in SW Washington. However, the mixed morph differs from red and black males in some, but not all, traits investigated. To review, we discovered that males from the mixed morph are intermediate in color relative to red and black males, but do not differ in color from either type

statistically (Figure 1.4). Similarly, the transmittance of light at 405 nm of the five mixed locations together does not differ from either red or black sites (Figure 1.3B). We also show that there are dramatic changes in habitat (Figure 1.1) and significant differences in water color (Figure 1.3D) within Connor Creek. Further, male color and shape are correlated, and this relationship differs between black and mixed males, but not between red and mixed males (Figure 1.5). Within the mixed morph, an LDA based on shape and color classified slightly more individuals in Connor Creek as “black” than “red” (Figure 1.6).

When assessing only shape, mixed males do not differ from red and black morphs in body shape (PC1) but do differ from both morphs in head shape (PC2), which is evident from the larger and more elongated head (Figure 1.8C). Even within Connor Creek, mixed males differed in shape from the black morph found further upstream (Figure 1.9). Mixed males were larger than both red and black males (Figure 1.10), had fewer lateral plates than red fish, but did not differ in lateral plating when compared to black fish. However, 4.6% of the sampled individuals were fully-plated, which we otherwise saw only at red freshwater sites. Full plating is also characteristic of the anadromous form (Bell 2001).

Together, the intermediate coloration, the variation in shape patterns, the increased size, and polymorphic plating relative to the red and black morphs all create a unique and perplexing story within Connor Creek. Although we are not certain how much of the measured variation in morphology and color reflects underlying genetic variation, many of the traits we examined are shown to be heritable (McPhail 1977; Aguirre et al. 2004) and have been genetically mapped (Peichel et al. 2001; Cresko et al. 2004; Schluter et al. 2004; Colosimo et al. 2005; Albert et al. 2008). Given the genetic basis of these traits, the larger size of anadromous stickleback relative to freshwater forms (Head et al. 2013), the similarity in nuptial coloration and body armor of the red freshwater morph and anadromous form (McKinnon and Rundle 2002; Bell 2001), as well as its proximity to the Pacific Ocean, it is possible that the phenotypic variation we observe in Connor Creek is the result of introgressive hybridization between anadromous stickleback and the black morph that resides further upstream.

Future work to determine the extent to which the color, shape, size, and plating traits we investigated are under ecological and/or sexual selection will illuminate how natural and sexual selection may interact to drive, maintain, or limit divergence among morphs in SW Washington. It would be interesting to know, additionally, if the phenotypic divergence that we observe between freshwater morphs is associated with genetic divergence among populations and color morphs, and if our hypothesis of an anadromous-freshwater hybrid zone in Connor Creek is also supported by genomic variation.

Chapter 2: Genetic divergence among threespine stickleback that differ in nuptial coloration

Introduction

Since Darwin first described the origin of species, the “mystery of mysteries” (1859), decades of research have unveiled a more complete understanding of the evolutionary mechanisms that reduce or prevent the exchange of genetic material between populations (gene flow; Mayr 1963; Coyne and Orr 2004; Schemske 2010; Sobel et al. 2010; Maan and Seehausen 2011), preventing or promoting the emergence of different species. Early explanations for reduced gene flow emphasized the roles of ecology and geography (Mayr 1947; Coyne and Orr 2004). When populations become geographically isolated, ecologically-mediated divergent selection and drift within isolated populations can drive phenotypic and genetic divergence without the homogenizing force of gene flow (Hatfield and Schluter 1999; Schluter 2001; Rundle and Nosil 2005; Nosil 2012). Darwin’s idea of natural selection is widely accepted, yet while he focused primarily on the variation observed within species, we have much to learn about the transition from divergence within species to divergence among species.

Polymorphic species have provided evolutionary biologists with important insights about the origin and maintenance of biodiversity (Schluter 2000; Hoekstra et al. 2004; Roulin 2004; Rueffler et al. 2006; Gray and McKinnon 2007). In the absence of selection maintaining multiple morphs, we should expect gene flow to erode differences between them (Mayr 1947; Slatkin 1987). Under what circumstances does within-species variation lead to differences between species? Nosil (2005) shows that reproductive isolation is greatest between populations of walking sticks (*Timena cristinae*) that differ in host-plant use when migration between hosts is low enough to prevent gene flow from eroding divergence. There can also be selection sufficient enough to promote speciation through the evolution of reproductive isolation between diverging

populations (Mayr 1947, 1963; Coyne and Orr 2004). This is exemplified, for instance, in the high rates of evolutionary diversification of cichlid fish in the African Great Lakes (Seehausen 2000) and in the classic example of adaptive radiation observed in Darwin's finches (Grant and Grant 2002). In contrast, we expect multiple discrete morphs to be maintained within species when their fitness is approximately equal, as with frequency-dependent selection (Slatkin 1979; Gadgil 1972). For example, in the marine isopod (*Paracerceis sculpta*), three genetically discrete male morphs coexist and do not differ in their reproductive success or survival despite their different morphologies and behaviors (Shuster and Wade 1991). However, the origin and maintenance of multiple morphs within a species remains a major question in evolutionary biology.

We capitalize on populations of the threespine stickleback fish (*Gasterosteus aculeatus*) from the northwest United States where two different male color morphs, red and black, are found, in some cases within a single stream or drainage (McPhail 1969; Hagen and Moodie 1979). We test for genetic isolation and levels of gene flow between the two color morphs where their distributions do not overlap and in one region where red and black stickleback have historically co-occurred. While much threespine stickleback research focuses on phenotypic and genetic divergence between species pairs (e.g. limnetic and benthic or marine and freshwater; reviewed in McKinnon and Rundle 2002), we characterize within-species and among-morph genetic variation to understand the evolution of genetic isolation in its earliest stages.

Threespine stickleback repeatedly and independently colonized freshwater coastal habitats in the northern hemisphere from marine environments at the end of the Pleistocene epoch, following the last glacial maximum less than 12,000 years ago (McPhail 1994). In ancestral marine and most derived freshwater locations, male threespine sticklebacks display a bright red throat during the breeding season (hereafter referred to as red sticklebacks; McPhail 1969; Semler 1971; Hagen and Moodie 1979) that is strongly preferred by females in mate choice (Semler 1971; Milinski and Bakker 1990; McKinnon 1995; Tinghitella et al. 2015). However, in several locations along the Pacific coast of North America, males have lost their iconic mating signal, and instead have full-body black breeding coloration (hereafter referred to as black

stickleback; McPhail 1969; Semler 1971; Reimchen 1989), an evolutionary shift best explained by differences in water color (Reimchen 1989; Scott 2001; Boughman 2001; Jenck et al. in prep). The two color morphs are maintained in common garden and black fish do not express red throats (unlike British Columbian benthics that are also sometimes described as “black”; Boughman 2001; Lewandowski and Boughman 2008), even when fed carotenoid rich diets (personal observation). In the limnetic-benthic species pair, the mating signals of red stickleback have high contrast in relatively clear water whereas black stickleback have high contrast on a background of red-shifted, tannin-rich water (Boughman 2001), making each color morph more visible in their respective environments. Females are relatively drab and do not express red throat coloration. When sexual signals shift in response to changes in their transmission environments (sensory drive; Endler 1992) it can lead to variation in reproductive success among types, ultimately leading to genetic divergence among populations (Fisher 1930; Panhuis et al. 2001; Boughman 2002; Servedio and Boughman 2017). Sites where red and black stickleback are found in the Pacific Northwest are largely allopatric (non-overlapping in distribution) but in pioneering work, McPhail (1969) and Hagen and Moodie (1979) documented several regions in southwest Washington where red and black stickleback have overlapping breeding grounds and seasons. One of these is Connor Creek, WA. We collected stickleback in Connor Creek nearly 50 years after these initial observations along a transect that mimicked McPhail's (1969). Contrary to historical records, the fish we collected at several sites were phenotypically intermediate in color and body shape to red and black fish from allopatric sites elsewhere in Washington, and were also phenotypically different from black fish further upstream in Connor Creek (hereafter referred to as mixed stickleback; Jenck et al. in prep). These observations and the outcome of mating trials described below raised questions about how much gene flow occurs between morphs, if any, and how this polymorphism is maintained in the absence of physical barriers to gene flow.

In polymorphic species, communication between males and females can play a critical role in isolation between morphs (Panhuis et al. 2001). When sexual selection plays a role in speciation, it is thought to do so when female preferences for specific mating signals reduce

mating between morphs or populations within a species, initiating reproductive isolation (Boughman 2001; Panhuis et al. 2001; Seehausen et al. 2008; van Doorn et al. 2009). Yet, given a choice, drab females from both color morphs direct more early courtship behaviors towards males with the ancestral red signal (Tinghitella et al. 2015), and, in no-choice mating trials between color morphs, there is no evidence for assortative mating, resulting in equal reproductive success of both male types (Tinghitella unpublished; McKinnon 1995; but see Scott 2004). Thus, female choice does not seem to be a driving force preventing the color morphs from interbreeding (Tinghitella et al. 2015). Though elaborate male mating signals and strong female preferences (inter-sexual selection) have been the dominant paradigm regarding sexual selection, recent work has emphasized the role of male competition (intra-sexual selection) in driving reproductive isolation, including between divergent stickleback types (Lackey and Boughman 2013, Keagy et al. 2016, Tinghitella et al. 2015, Tinghitella et al. 2018a, reviewed in Tinghitella et al. 2018b). In this study system, in simulated secondary contact, black males bias their aggression toward red males while red males show no bias in aggressive behavior, making red males the recipients of more aggression overall (Tinghitella et al. 2015). Given that more aggressive males are more likely to establish territories (Tinghitella et al. 2018a), the observed bias in competition behaviors could contribute to habitat isolation between the two color morphs, and thus reduced gene flow. The conflicting sexual selection patterns observed (male competition contributing positively to isolation, but female choice not) further prompted us to investigate the potential for genetic isolation between the two color morphs.

Here, we adopt both broad scale (among sites) and fine scale (within a site) approaches to learn the extent to which stickleback that differ in nuptial coloration are genetically distinct from one another. We assess genetic divergence and estimate gene flow across a relatively wide geographic scale that consists of tributaries containing several red and black sites. Because the maintenance of multiple morphs in the absence of physical barriers to gene flow is still a heavily debated topic, we also assess intraspecific divergence and estimate gene flow between mixed stickleback, which are phenotypically intermediate to the red and black morphs, at a finer

geographic scale, in a single stream where historical records indicate coexistence of the two color morphs (McPhail 1969; Hagen and Moodie 1979). Here, there are no geographic barriers preventing mating between morphs, thus in the absence of other barriers, we expect unimpeded gene flow and breakdown in any divergence between morphs. Yet, there are no previous reports of hybridization in this region (McPhail 1969; Hagen and Moodie 1979). If prezygotic (behavioral isolation) or postzygotic (reduced viability of hybrids) reproductive barriers have evolved between color morphs, we should instead find that gene flow is limited and that red and black stickleback maintain genetic isolation by color. We used double digest restriction-site associated DNA-sequencing (ddRAD-seq; Peterson et al. 2012; Catchen et al. 2013) to characterize partitioning of genetic variation among and within 14 sites where red stickleback only, black stickleback only, or mixed stickleback are found. The system has the potential to yield new insights into how phenotypic and genetic differentiation is maintained and whether evolution within a species can give rise to new species in the absence of assortative mating.

Methods

Collection of samples

We collected sexually mature, adult stickleback from streams and rivers of southwest Washington (WA) and western Oregon (OR), US, and fin clipped them in the field or transferred them to the University of Denver (followed by tissue sampling) during the summers of 2016-2017 (Figure 2.1, Table 2.1).

Figure 2.1.

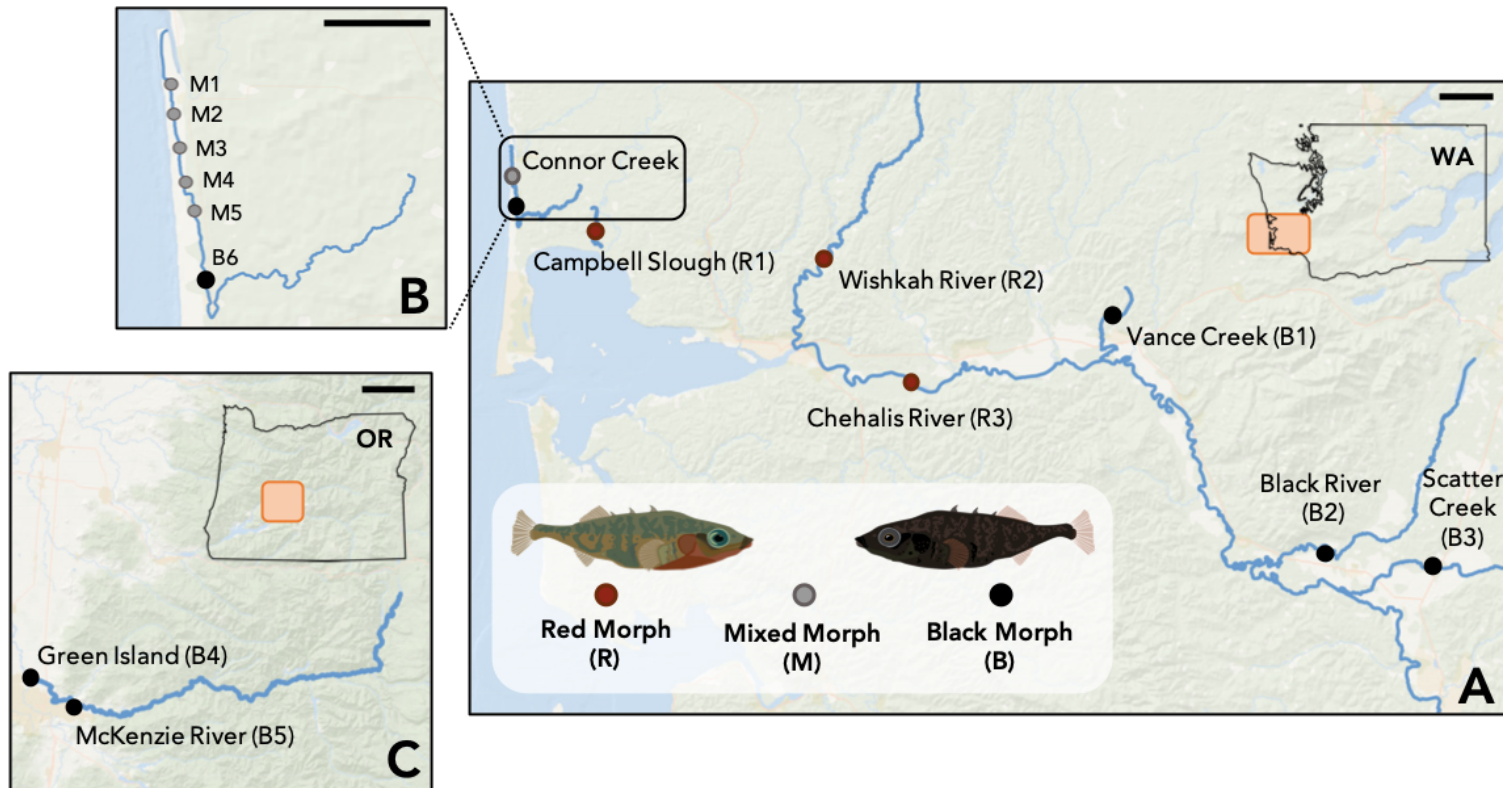


Figure 2.1. Threespine stickleback morphs and collection sites in Washington (A,B) and Oregon (C). Connor Creek collection sites (B) mirror those of McPhail (1969). Sites where we collect red stickleback are denoted with “R”, sites where we collect black stickleback are denoted with “B”, and sites where we collect mixed stickleback are denoted with “M” for mixed. All black bars in the top right of each panel correspond to a distance of 5 kilometers.

Table 2.1. Sites collected, color morph found in each site, GPS coordinates of collection sites, collection dates, and the number of individuals genotyped. Red sites are denoted with “R”, black sites are denoted with “B”, and mixed sites are denoted with “M”.

Table 2.1.

Sites	Color Morph	GPS Coordinates	Collection Date(s)	Genotyped
Campbell Slough (R1)	Red	47°2'40"N, 124°3'33" W	May 2016	32
Wishkah River (R2)	Red	47°0'17"N, 123°48'49"W	May 2018	11
Chehalis River (R3)	Red	46°56'22"N, 123°18'46"W	May 2016	29
Vance Creek (B1)	Black	46°59'48"N, 123°24'43"W	May 2016	20
Black River (B2)	Black	46°49'45"N, 123°8'1"W	May 2016	28
Scatter Creek (B3)	Black	46°49'20"N, 123°3'11"W	May 2016	27
McKenzie River (B4)	Black	44°3'41"N, 122°51'11"W	June 2017	9
Green Island (B5)	Black	44°8'42"N, 123°7'5"W	June 2017	11
Connor Creek (B6)	Black	47°4'11"N, 124°10'5"W	May 2016	25
Connor Creek (M1)	Mixed	47°6'55"N, 124°10'52"W	May 2018	46
Connor Creek (M2)	Mixed	47°6'26"N, 124°10'45"W	May 2018	11
Connor Creek (M3)	Mixed	47°5'57"N, 124°10'39"W	May 2018	17
Connor Creek (M4)	Mixed	47°5'29"N, 124°10'30"W	May 2018	3
Connor Creek (M5)	Mixed	47°5'12"N, 124°10'20"W	May 2018	27

We include two sites in Oregon that were not glaciated during the Pleistocene epoch (Catchen et al. 2013) to serve as an outgroup and to assess the relatedness of black fish across sites that differ dramatically in their colonization times (OR: several million years, WA: ~12,000 years). Fish with ancestral, red nuptial coloration were collected from three sites (Campbell Slough (R1), Wishkah River (R2), and Chehalis River (R3)), and fish with black nuptial coloration were collected from six sites (Vance Creek (B1), Black River (B2), Scatter Creek (B3), McKenzie River (B4), Green Island (B5), and Connor Creek (B6)). Each of these collection sites are allopatric regions where either the red or the black color morph exist. We collected stickleback using non-baited, galvanized steel mesh minnow traps. In summer 2018, we also conducted a finer scale sampling regime within Connor Creek where the two color morphs have historically coexisted (McPhail 1969; Hagen and Moodie 1979). Within Connor Creek, we sampled five locations by paddleboarding along a 3.5 kilometer-long transect, trapping at approximately 0.9-kilometer intervals, beginning near the mouth of the creek (M1) and moving further inland toward our 2016 Connor Creek sampling site where only black fish are found (B6; Figure 2.1). We dropped a total of 35 minnow traps in the creek with five to 10 traps set in each location along the transect, depending on available breeding grounds. This collection was intended to parallel the one first done by McPhail (1969). Following the retrieval of traps, the fish that were being transferred back to the lab (for behavioral experiments not reported here) were sorted, packed, and shipped directly to the lab as air cargo (next flight guaranteed). We released fish that were only collected for genotyping immediately after fin clipping.

Ultimately, we genotyped a total of 296 individuals from 14 sites, 127 of which are from the fine scale sampling of Connor Creek (Table 2.1). For fish that were used in lab experiments, we collected tissue samples following their natural death in the lab. Fish were fixed in jars containing 90% ethanol that were separated by site. We collected all of the caudal fin and muscle tissue up to the posterior end of the ventral fin using sterile techniques between individuals. For fish that were sampled in the field, we cut no more than half of the caudal fin (R2, B4, B5, and

Connor Creek individuals collected in 2018 (M1-M5)). Upon collection, we immediately placed each sample in 90% ethanol.

Library preparation

To extract genomic DNA, we used the DNeasy Blood and Tissue Kit (Qiagen®), following the manufacturer's protocol. We prepared RAD libraries following the double digest RAD-seq protocol in Peterson et al. (2012), with the following modifications. After the extraction of DNA, we quantified DNA concentration using the Quantifluor® dsDNA System (Promega) and set up the double digestion using *EcoRI* and *MspI* restriction enzymes (New England Biolabs® Inc.). Using the package SimRAD (version 0.96; Lepais and Weir 2014) in R (R Core Development Team 2010) and the *G. aculeatus* genome assembly (Peichel et al. 2017), we chose the restriction enzymes based on the best number of loci that each combination of enzymes could yield, which was calculated for fragments of 300 ± 24 base pairs. We cleaned the digested samples using homemade Seramega beads (produced following Rohland and Reich 2012) and quantified DNA concentrations again using the Quantifluor® dsDNA System. We then performed adapter ligation on the digested samples using up to 19 unique barcodes (Integrated DNA Technologies Inc.). Whenever possible, individuals from each site were divided across four library preps to avoid batch effects; the exception is with fish collected in 2018 (sites M1-M5 and R2). After the samples were pooled by index and cleaned, we size selected fragments of 300 ± 24 base pairs in length using Bluepippin size selection at the BioFrontiers Institute Next-Gen Sequencing Core Facility, University of Colorado Boulder. We amplified each size selected library in three 20 μ L reactions using Phusion® High-Fidelity DNA Polymerase (New England Biolabs® Inc.) to integrate uniquely indexed PCR sequences (Integrated DNA Technologies Inc.) to all fragments. Finally, we cleaned and quantified the amplified libraries as previously described before they were pooled to compose the final libraries. We single-end sequenced four libraries using an Illumina NextSeq 500 at the BioFrontiers Institute Next-Gen Sequencing Core Facility.

Population genomics

We demultiplexed and trimmed the resulting raw sequences with *process_radtags* in the Stacks software pipeline (version 1.46; Catchen et al. 2013) using our unique barcodes. We used the default filtering of *process_radtags* to discard reads with low quality scores (phred score) and reads with uncalled bases; reads with a quality score below a 90% probability of being correct (phred score of 10), with a sliding window of 15% of the length of the read, were removed. We aligned these processed reads to the revised threespine stickleback genome (Peichel et al. 2017) using BWA-MEM (version 0.7.12; Li 2013). We then identified and called single-nucleotide polymorphisms (SNPs) throughout the genome using Freebayes (version 1.2.0; Garrison and Marth 2012) to create a VCF catalog of SNPs from all individuals and sites. We used VCFtools (version 0.1.16; Danecek et al. 2011) to remove individuals missing more than 60% of loci, to include only bi-allelic SNPs across all individuals, to exclude SNPs that are not present in 85% of individuals, to exclude all genotypes with a quality below a threshold of 20, and to exclude all genotypes that do not meet a minimum depth of five and a maximum depth of 200. Using this VCF catalog containing filtered SNPs across all individuals from all sites, we conducted all population genomic analyses described below with three different datasets: the first contained individuals from all sites in both WA and OR (R1-R3, B1-B6, M1-M5), the second contained individuals from all WA sites only (excluding OR; including R1-R3, B1-B3, B6, M1-M5), and the third contained individuals from sites within Connor Creek only (B6, M1-M5).

First, we used principal component analyses (PCAs) to identify and display possible groupings and patterns of individuals across sites with the package *SNPRelate* (version 1.16.0; Zheng et al. 2012) in R. We then calculated pairwise F_{ST} values (Weir and Cockerham 1984) to assess the extent of genetic variation among sites using the package *genepop* (version 1.1.2; Rousset 2008) in R. To visualize the hierarchical relationships among clusters, we also created a dendrogram by performing a hierarchical cluster analysis and standardizing variability among individuals with z-scores using *SNPRelate*. To define the genetic structure of individuals across sites for each of the three datasets, we first used *STRUCTURE* (version 2.3.4; Pritchard et al.

2000) to analyze differences in the distribution of genetic variants among sites. For the dataset containing all individuals from all sites, possible K values ranged from one to nine with five replicates per K value. For the dataset containing individuals only from sites in WA, possible K values ranged from one to seven with five replicates per K value. Lastly, for the dataset containing only individuals from Connor Creek, possible K values ranged from one to six with five replicates per K value. All STRUCTURE runs were performed with a burnin period of 10,000 followed by 20,000 Markov Chain Monte Carlo repetitions. Following all STRUCTURE analyses, we used Structure Harvester (version 0.6.94; Earl and vonHoldt 2012) to identify the best value of K for each dataset that captures the uppermost level of genetic structure (implementing the Evanno method; Evanno et al. 2005). Finally, we used CLUMPAK (Kopelman et al. 2015) to produce graphical displays of STRUCTURE results.

Results

We sequenced 371 individuals from 14 sites spread across four libraries using four lanes in a NextSeq 500 Illumina sequencer. Of the 788,037,686 total raw reads generated, we retained 74.80% in the first library, 98.15% in the second, 95.80% in the third, and 31.31% in the fourth following demultiplexing and dropping of low quality reads, ambiguous barcode reads, and ambiguous RAD-Tag reads. Following the alignment of processed reads to the genome, calling SNPs across all individuals, and filtering the catalog of SNPs (see methods), we retained 296 of the 371 individuals and 3,304 of a possible 108,668 SNPs in the largest dataset that contained individuals from all collection sites in both WA and OR. In the second dataset that included only individuals from sites in WA, we retained 276 of the possible 347 individuals and 3,562 of the 108,668 possible SNPs. Finally, in the third dataset including individuals from sites only within Connor Creek, we kept 127 of the 152 individuals and 11,567 of the 108,668 possible SNPs.

Population genomics

In our PCA of the 296 individuals from sites in both WA and OR, we found that there is genetic divergence by distance and by color morph (Figure 2.2A).

Figure 2.2.

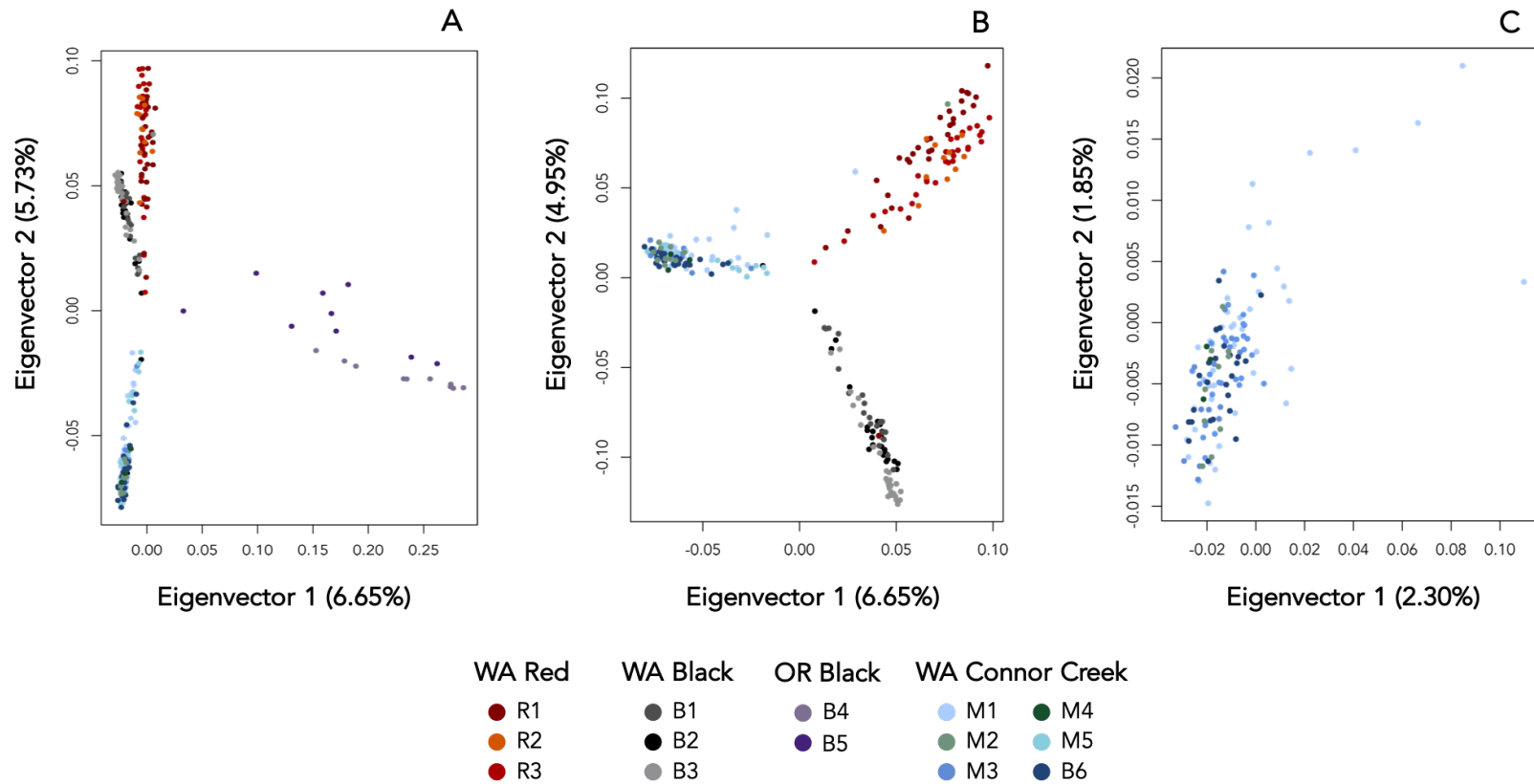


Figure 2.2. Principal component analysis of 296 individuals from all sites in Washington and Oregon (R1-R3, B1-B6, M1-M5; A), of 276 individuals from sites in only Washington (R1-R3, B1-B3, B6, M1-M5; B), and of 127 individuals from sites only within Connor Creek (M1-M5, B6; C).

The first major axis of genetic variation, eigenvector 1, explained 6.65% of the total variation and appears to isolate clusters by geographic distance, such that individuals from sites in OR are separated from individuals from sites in WA. The second major axis of genetic variation, eigenvector 2, explained 5.73% of the total variation and appears to isolate clusters by color, such that individuals we categorize as “red” are grouped together, individuals we categorize as “black” are grouped together, individuals we categorize as “mixed” are grouped together, and all clusters are clearly separated from one another. The PCA of the 276 individuals from sites only in WA confirmed genetic divergence among the red, black, and mixed color morphs (Figure 2.2B). The first major axis of genetic variation, eigenvector 1, explained 6.65% of the total variation and separates individuals belonging to the mixed color morph from individuals belonging to both the red and black morphs. The second major axis of genetic variation, eigenvector 2, explained 4.95% of the total variation largely separates the red morph from the black morph, and interestingly, a cluster of individuals belonging to the mixed morph falls between the red and black clusters. In our PCA of the 127 individuals from sites only within Connor Creek, we found that there is little to no genetic divergence among individuals, even between the black individuals at B6 and mixed individuals at sites M1-M5 (Figure 2.2C), which are 2.0 (B6 to M5) to 5.5 (B6 to M1) kilometers away. Eigenvector 1 and eigenvector 2 explained 2.30% and 1.85% of the total genetic variation, respectively.

Our measures of differentiation also demonstrate large amounts of divergence among sites and mirror the findings displayed in all three PCAs (Table 2.2).

Table 2.2. Wier and Cockham's F_{ST} pairwise comparisons for all sites.

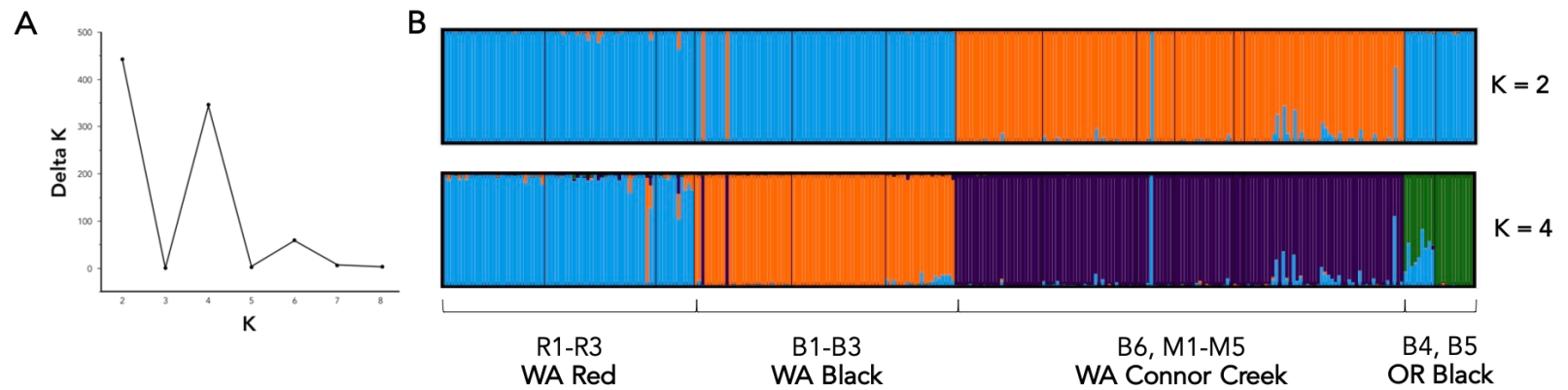
Table 2.2.

	R2	R3	B1	B2	B3	B4	B5	B6	M1	M2	M3	M4	M5
R1	0.027	0.0025	0.31	0.31	0.39	0.56	0.34	0.37	0.34	0.31	0.36	0.34	0.36
R2		0.012	0.27	0.28	0.36	0.51	0.30	0.33	0.29	0.26	0.31	0.28	0.32
R3			0.28	0.28	0.39	0.58	0.32	0.36	0.31	0.28	0.35	0.31	0.34
B1				0.010	0.11	0.59	0.39	0.34	0.30	0.29	0.33	0.32	0.33
B2					0.11	0.58	0.40	0.32	0.29	0.28	0.32	0.31	0.31
B3						0.67	0.50	0.42	0.38	0.40	0.43	0.44	0.42
B4							0.18	0.57	0.52	0.56	0.58	0.66	0.56
B5								0.39	0.36	0.33	0.38	0.34	0.38
B6									0.0043	0.0040	0.0020	-0.0024	0.0025
M1										0.0013	0.0015	-0.0122	0.0021
M2											0.0038	-0.019	0.0020
M3												0.0027	-0.0021
M4													0.0017

F_{ST} pairwise comparisons among WA red sites varied from 0.0025 to 0.027, indicating low genetic differentiation within the red color morph. However, all WA red sites are relatively divergent from WA black sites (F_{ST} values ranging from 0.27 to 0.39) and from WA mixed sites (F_{ST} values ranged from 0.26 to 0.36), and even more so from OR black sites (F_{ST} values ranged from 0.30 to 0.58). Pairwise F_{ST} among WA black sites varied from 0.010 to 0.42. However, if we exclude the black site in Connor Creek (B6) from this group, F_{ST} values only varied from 0.010 to 0.11 among black sites within WA. Genetic differentiation is greater within the WA black color morph relative to the red morph. All WA black sites are relatively divergent from WA red sites, from OR black sites (F_{ST} values ranged from 0.39 to 0.67), and from WA mixed sites (excluding B6, F_{ST} values ranged from 0.28 to 0.44). Similar to the WA black morph, the F_{ST} pairwise comparison between OR black sites, 0.18, indicates higher genetic differentiation within the OR black color morph relative to other morphs. Additionally, OR black sites are relatively divergent from WA red sites, WA black sites, and WA mixed sites (F_{ST} values ranged from 0.33 to 0.66). F_{ST} pairwise comparisons among all sites within Connor Creek, including B6, varied from -0.019 to 0.0043. This suggests that there is little genetic subdivision in Connor Creek, even between the black and mixed morphs. Though we find minimal divergence, it is interesting to note that the largest pairwise comparison in this range, 0.0043, is between the two sites that are the furthest apart (M1 and B6). All Connor Creek sites are largely differentiated from all WA red sites, WA black sites, and OR black sites.

STRUCTURE analyses produced results consistent with the PCAs and F_{ST} pairwise comparisons. With the delta K method (Evanno et al. 2005), we found that a model using $K = 2$ best fits the dataset of individuals from all sites in WA and OR, and $K = 4$ is the next best value to describe their genetic structure (Figure 2.3A).

Figure 2.3.



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Figure 2.3. STRUCTURE analysis of individuals from all Washington and Oregon sites with cluster assignments based on the two highest calculated delta K values (A): K = 2 (top; B) and K = 4 (bottom; B). Each colored vertical bar represents a single individual within a site, wherein the proportion of each color represents the likelihood of membership to each cluster. Collection sites are separated by black vertical bars.

The STRUCTURE plot created with $K = 2$ showed that together, individuals from WA red sites, individuals from WA black sites, and individuals from OR black sites, were distinguished from individuals from WA mixed sites in Connor Creek (Figure 2.3B; top). Increasing the value of K to four provided additional resolution of population structure in which individuals from OR black sites were assigned to a different, separate cluster (Figure 2.3B; bottom). We found that a model using $K = 2$ again best fits the dataset of individuals from sites in only WA, and $K = 3$ is the next best value to describe their genetic structure (Figure 2.4A).

Figure 2.4.

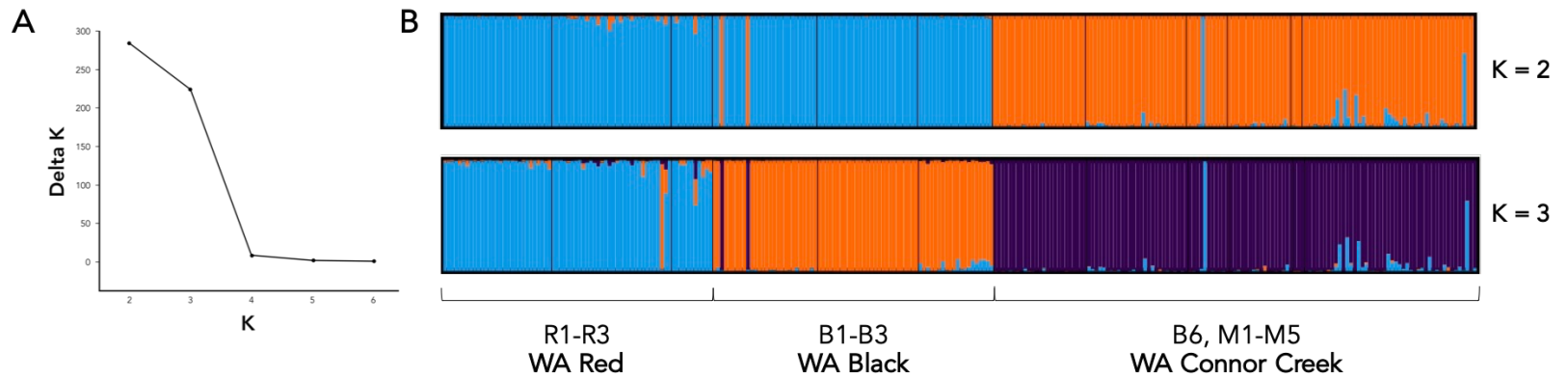


Figure 2.4. STRUCTURE analysis of individuals from all Washington sites with cluster assignments based on the two highest calculated delta K values (A): K = 2 (top; B) and K = 3 (bottom; B). Each colored vertical bar represents a single individual within a site, wherein the proportion of each color represents the likelihood of membership to each cluster. Collection sites are separated by black vertical bars.

Similar to the dataset that includes OR individuals, the STRUCTURE plot created with $K = 2$ separated individuals from WA red sites plus WA black sites from individuals from WA mixed sites (Figure 2.4B; top). Increasing the value of K to three produced a plot in which individuals from WA red sites and individuals from WA black sites were separated and each was also distinct from the group of WA mixed individuals (Figure 2.4B; bottom). Lastly, we found that a model using $K = 2$ best fit the dataset of individuals from the black site (B6) and mixed sites (M1-M5) in Connor Creek (Figure 2.5A).

Figure 2.5.

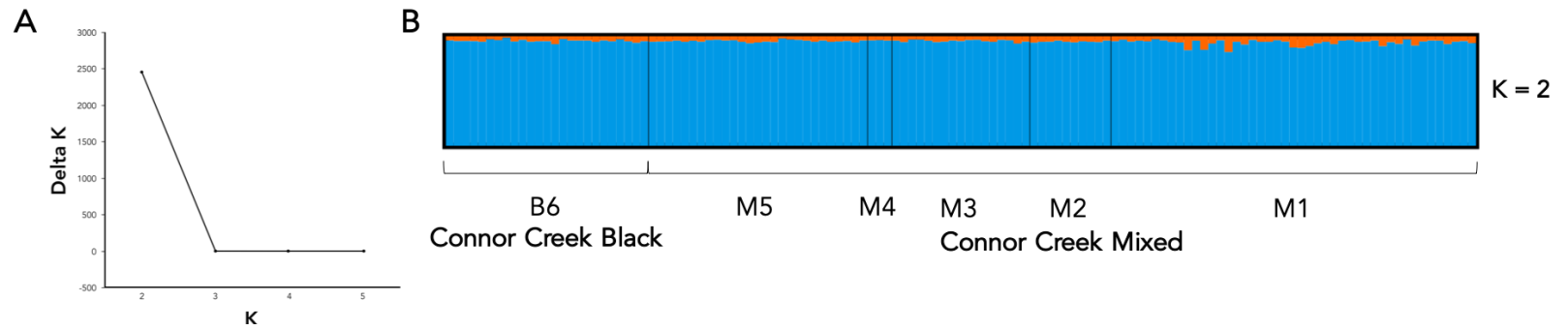


Figure 2.5. STRUCTURE analysis of individuals from sites within Connor Creek with cluster assignments based on the highest calculated delta K value (A), $K = 2$ (B). Each colored vertical bar represents a single individual within a site, wherein the proportion of each color represents the likelihood of membership to each cluster. Collection sites are separated by black vertical bars.

In accordance with the PCA (Figure 2.2C), the STRUCTURE plot created with $K = 2$ showed that there is no apparent genetic differentiation or structure among individuals from different sites within Connor Creek (Figure 2.5B).

Finally, our hierarchical cluster analysis produced four well-supported groups that generally parallel the results shown in the PCAs, F_{ST} pairwise comparisons, and STRUCTURE analyses, and the dendrogram further illustrates the relationships between these groups (Figure 2.6).

Figure 2.6.

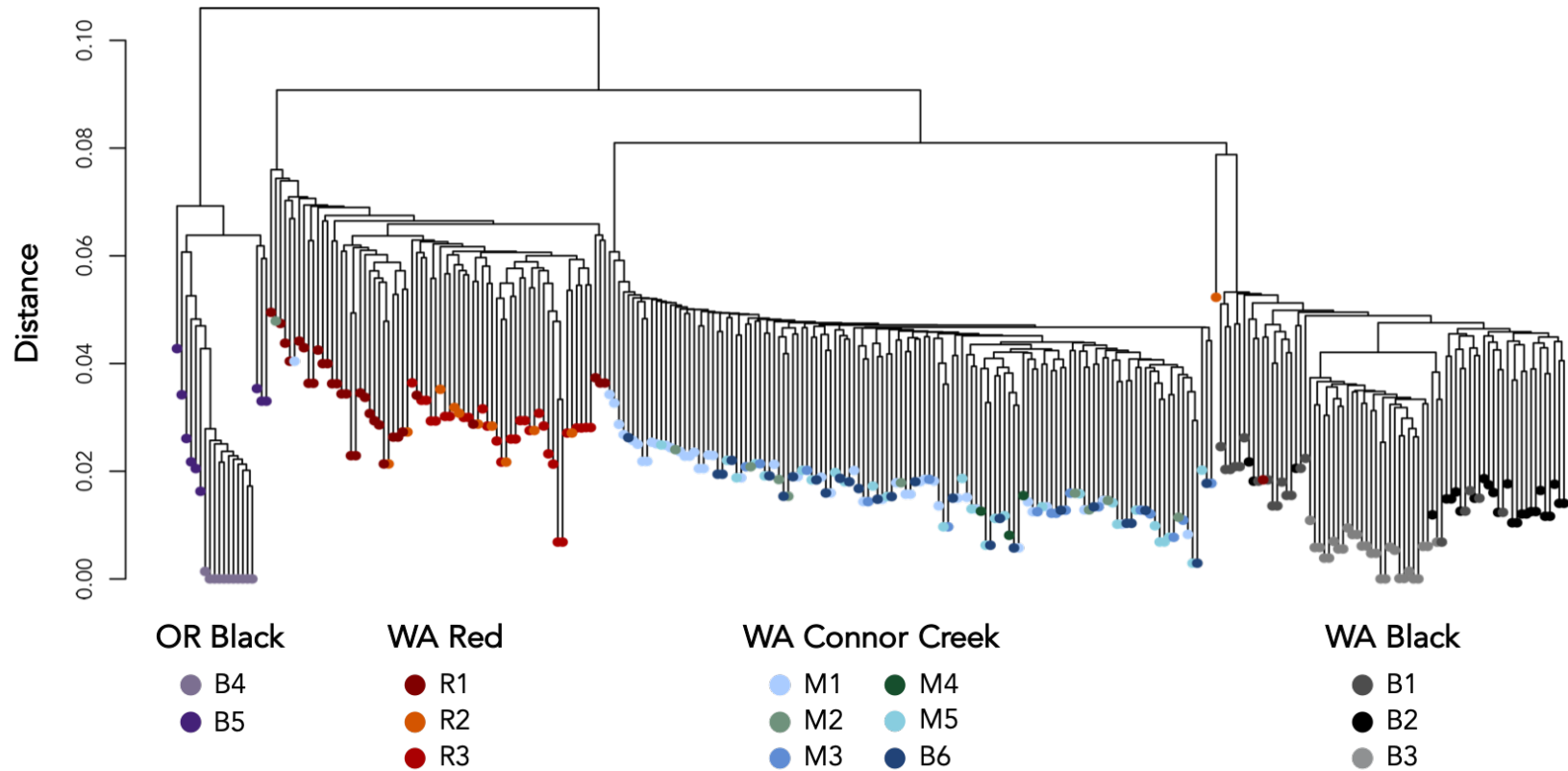


Figure 2.6. Dendrogram displaying subgroups of individuals determined by hierarchical cluster analysis. The y-axis, distance, represents the closeness, or similarity, of individuals and clusters.

Most notably, we found that there were two major branches in the dendrogram, in which the outgroup clade comprised of individuals from the OR black sites was the most genetically different from the clade comprised of individuals from all WA sites. Within the WA clade, individuals from WA red sites were more genetically distinct from individuals from WA black and WA mixed sites. There were no apparent groups within the WA red cluster and individuals were intermixed, suggesting low genetic differentiation within the red morph. Similarly, there were no apparent groups within the WA mixed cluster and individuals were intermixed, suggesting low genetic differentiation within the mixed morph. However, the dendrogram revealed greater resolution than suggested in the other analyses and we found that there were apparent groups by site within the WA black cluster; individuals from B1 were grouped together, individuals from B2 were grouped together, and individuals from B3 were grouped together. There were also apparent groups within the OR black cluster; individuals from B4 were grouped together and individuals from B5 were grouped together.

Discussion

We examined genome-wide genetic variation among morphs of threespine stickleback in the Pacific Northwest that recently diverged in nuptial coloration. In allopatric regions of their distribution, the red and black morphs differ in nuptial color, body shape, and bony plating (Jenck et al. in prep), yet appear to interbreed freely in simulated secondary contact (McKinnon 1995; Tinghitella et al. 2015, 2018a, unpublished). We characterized the genetic structure among these morphs using ddRAD-seq data, finding clear evidence of genetic divergence between red and black stickleback across sites in Washington and Oregon. We also found that a “mixed” morph in Connor Creek (phenotypically intermediate in color and body shape characteristics) is genetically divergent from both the red and black WA morphs.

Genetic divergence between Washington and Oregon

When we consider the broadest geographic extent of our sampling, the greatest axis of genetic divergence in stickleback inhabiting freshwater habitats of Washington and Oregon is one

that separates fish from the two states (Figure 2.2A). This pattern is supported by the high values of the F_{ST} pairwise comparisons between all Washington and Oregon sites (Table 2.2), by separation along eigenvector 1 of our PCA of the full dataset (Figure 2.2A), and by separation of Oregon from all Washington clusters depicted in the dendrogram (Figure 2.6). It is possible that this genetic divergence may be shaped by geographic distance. Geographically distant populations tend to be more genetically differentiated than nearby populations (Wright 1943), and patterns of isolation-by-distance have been detected between stickleback inhabiting even contiguous lake and stream habitats on Vancouver Island, British Columbia (Weber et al. 2017). However, the STRUCTURE output of the best K value for this dataset ($K = 2$; Figure 2.3B; top), does not separate black Oregon fish from red or black Washington fish. Notably, Oregon fish are only separated from all Washington fish with the next best value of K ($K = 4$; Figure 2.3B; bottom). Much of what is presently Oregon was not glaciated during the last glacial maximum and many aquatic habitats, specifically those inland, are much older than northern or coastal ones (O'Connor 2001; Booth et al. 2003). Thus, stickleback populations that inhabit sites we sampled in Oregon precede the last glacial maximum and are millions of years older than those we sampled in Washington (Catchen et al. 2013), which colonized freshwater habitats following glacial retreat less than 12,000 years ago (McPhail 1994). Though isolation-by-distance may contribute to the genetic divergence we observe between Washington and Oregon, differences in geographic history between the two states may explain why Oregon individuals are the most genetically differentiated – the outgroup of the dendrogram – relative to Washington individuals (Figure 2.6). Because we only sampled two sites in Oregon that were in close proximity to one another, it would be beneficial for future studies to more extensively sample red and black stickleback from this region to add to this dataset, as well as marine stickleback to serve as a representative of freshwater stickleback ancestors.

Genetic divergence among morphs in Washington

When we consider a narrow geographic range of sites only in Washington, the greatest difference in genetic variation is the partition between the red and black morphs together, and all

individuals in Connor Creek (Figure 2.2B). This is also reflected in the STRUCTURE output ($K = 2$), in which Connor Creek is isolated from the other two morphs (Figure 2.4B; top). Thus, there appears to be stronger divergence between stickleback in Connor Creek and the group of red plus black morphs, than there is between the red and black morphs. Notably, the sampling locations in Connor Creek approach the mouth of the creek, where it is likely that freshwater and anadromous stickleback come into contact.

Within Washington, the next major axis of genetic variation is between the red and black morphs (Figure 2.2B). Both PCA and STRUCTURE ($K = 3$) separate the three morphs from one another and our measures of differentiation also indicate that there is strong and approximately equal divergence among all three morphs, suggesting that gene flow is limited among color morphs. F_{ST} pairwise comparisons ranged from 0.27 to 0.39 between red and black morphs, 0.26 to 0.36 between red and mixed morphs, and 0.28 to 0.44 between the black and mixed morphs (Table 2.2). Similar F_{ST} values have been reported in freshwater, inland populations in Oregon that differ in morphology (ranging from 0.33 to 0.37; Currey et al. 2019). The genetic variation among these three morphs closely mirrors their phenotypic variation, wherein the mixed morph falls intermediate to the red and black morphs in color and body shape (Jenck et al. in prep).

Genetic divergence within morphs

While there is likely little gene flow among color morphs, divergence is not as evident among sites within each color morph. We found that within the red morph, both F_{ST} pairwise comparisons and the hierarchical cluster analysis suggest substantial gene flow among red sites, as individuals are not clustered by site on the dendrogram (Figure 2.6), which also reflects the low F_{ST} values among red sites (Table 2.2). Similarly, there is no genetic divergence and high levels of gene flow among individuals within Connor Creek; individuals do not cluster by site on the dendrogram (Figure 2.6), and this reflects the low values of differentiation among sites in Connor Creek (Table 2.2) as well as the STRUCTURE analysis of only Connor Creek individuals (Figure 2.5B). It is possible that the geographic distances studied are insufficient to prevent migration and/or interbreeding across sites as freshwater stickleback can travel up to five

kilometers to breeding sites (Snyder and Dingle 1989). Extensive gene flow is particularly likely among mixed sites as each collection site resides along a 3.5-kilometer-long transect within a single creek and we did not observe physical barriers to gene flow during collection.

However, relative to the red and mixed morphs, there are lower levels of gene flow among sites within the Washington black morph (excluding B6 within Connor Creek) and among sites within the Oregon black morph, indicated by the higher F_{ST} values (Table 2.2) and site-level clustering of individuals on the dendrogram (Figure 2.6). The cluster analysis in particular, suggests that Vance Creek (B1) likely originated and was established first followed by the colonization of Black River (B2) and then Scatter Creek (B3). The evolutionary switch from red to black nuptial coloration occurred after the invasion of freshwater habitats by marine stickleback following glacial retreat and, remarkably, Vance Creek (B1) is the black site closest to the ocean whereas Scatter Creek (B3) is the furthest away. This further supports that stickleback may have invaded Vance Creek (B1) first and later colonized new locations further inland, accumulating genetic divergence with geographic distance. Additionally, our black collection sites (excluding B6) are physically isolated from one another by the Chehalis River (R3) which is a large, fast moving river with relatively clear water, where we find red stickleback (Figure 2.1). It is interesting to note that the genetic divergence we observed exists despite no evidence for assortative mating in this system (McKinnon 1995; Tinghitella et al. 2015, unpublished). While sexual selection through female choice does not appear to drive divergence between color morphs, there is evidence in this system that male competition patterns may contribute to divergence (Tinghitella et al. 2015, Tinghitella et al. 2018a). In simulated secondary contact black males bias their competitive behaviors toward red males, a pattern that could contribute to habitat isolation between morphs. If red and black stickleback come into secondary contact in locations where black sites (B1-B3) meet the Chehalis River (R3), it is possible that ecological selection and male competition work together to yield genetic isolation between red and black stickleback.

Accumulation of evidence for Connor Creek as a potential hybrid zone

Despite the overall pattern of genomic divergence by color morph, our analyses of genetic structure showed that red and black stickleback from non-Connor Creek sites in Washington are more closely related to black stickleback in Oregon than they are to stickleback in Connor Creek (Figure 2.3B; top). This is despite the geographic proximity of red and black sites to Connor Creek, and the dramatic difference in age between Washington and Oregon stickleback. We also found that among individuals only in Washington, both PCA and STRUCTURE isolate Connor Creek from a grouping that includes both red and black sites (Figures 2.2B and 2.3B). We previously demonstrated that the mixed morph (M1-M5) differs phenotypically from the red and black morphs in some important ways; mixed fish are intermediate to the red and black color morphs in body shape and color, larger in size than red and black fish, and have fewer bony lateral plates than red fish (but still often express 'full' plating; Jenck et al. in prep). Curiously, the freshwater red morph shares traits that are characteristic of anadromous stickleback (similar color and plating; McKinnon and Rundle 2002; Bell 2001) but anadromous fish are larger than freshwater (Head et al. 2013). This leads us to hypothesize that in sites M1-M5 within Connor Creek, the mixed morph may be a consequence of interbreeding between phenotypically black fish and anadromous fish. Regions of overlap between divergent forms are characterized by high levels of gene flow, which gives rise to hybrid zones (Barton and Hewitt 1989). These contact zones are often found at environmental transitions and across ecological gradients (Endler 1986), and given how frequently marine and freshwater environments come into contact, it is not surprising that hybrid zones between freshwater-resident and anadromous stickleback are widespread (McPhail 1994; Jones et al. 2006; Hendry et al. 2009). It is feasible that the major partitioning of genetic variation we see among Washington stickleback is between freshwater (red and black morphs) and anadromous-freshwater hybrids (mixed morph). The partitioning of genetic variation within Connor Creek does not exactly mirror the partitioning of phenotypic variation, however. Genetic variation suggests extensive gene flow throughout Connor Creek, yet individuals from the five mixed sites (M1-M5)

were phenotypically different from their upstream neighbor (B6) in color and body shape (Jenck et al. in prep). We previously found evidence of both habitat differences (sandy bottom versus highly vegetated habitats inland) and changes in the transmission properties of the environment, wherein the water from sites further inland in Connor Creek are red-shifted and tannin-rich; these differences are consistent with selection for black coloration at B6 through sensory drive (Jenck et al. in prep).

Understanding the origin of species has been instrumental to the field of evolutionary biology. With the application of genomic approaches in natural populations, uncovering the genetic basis of multiple morphs and speciation is within reach. Although our measures of genome-wide divergence and genetic structure were largely consistent in their assignment of individuals to their respective morphs, future work is needed to better develop our understanding of the genes underlying diversity in this stickleback species pair. Additionally, the sampling regime discussed above would allow us to more definitively determine if black morphs evolved repeatedly upon freshwater invasions, and including marine fish representative of freshwater ancestors would further support our hypothesis of an anadromous-freshwater hybrid zone in Connor Creek. Our findings also lay the foundation for further investigation of the mechanisms responsible for driving genetic divergence and limiting gene flow, such as geographic or behavioral isolation, between stickleback color morphs, as well as those that drive phenotypic divergence despite an absence of barriers to gene flow.

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