
Article

Testing the potential mechanisms for the maintenance of a genetic color polymorphism in bluefin killifish populations

Ashley M. JOHNSON[†], Chia-Hao CHANG^{†,*}, and Rebecca C. FULLER

Department of Animal Biology, School of Integrative Biology, University of Illinois at Urbana-Champaign, Champaign, IL 61820, USA

*Address correspondence to Chia-Hao Chang. E-mail: chiahao0928@gmail.com.

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[†]These authors contributed equally to this work.

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Abstract

The maintenance of genetic variation in the face of natural selection is a long-standing question in evolutionary biology. In the bluefin killifish *Lucania goodei*, male coloration is polymorphic. Males can produce either red or yellow coloration in their anal fins, and both color morphs are present in all springs. These 2 morphs are heritable and how they are maintained in nature is unknown. Here, we tested 2 mechanisms for the maintenance of the red/yellow color morphs. Negative frequency-dependent mating success predicts that rare males have a mating advantage over common males. Spatial variation in fitness predicts that different color morphs have an advantage in different microhabitat types. Using a breeding experiment, we tested these hypotheses by creating populations with different ratios of red to yellow males (5 red:1 yellow; 1 red:5 yellow) and determining male mating success on shallow and deep spawning substrates. We found no evidence of negative frequency-dependent mating success. Common morphs tended to have higher mating success, and this was particularly so on shallow spawning substrates. However, on deep substrates, red males enjoyed higher mating success than yellow males, particularly so when red males were rare. However, yellow males did not have an advantage at either depth nor when rare. We suggest that preference for red males is expressed in deeper water, possibly due to alterations in the lighting environment. Finally, male pigment levels were correlated with one another and predicted male mating success. Hence, pigmentation plays an important role in male mating success.

Key words: carotenoid, color polymorphism, environmental heterogeneity, melanin, negative frequency dependence, pterin.

The ubiquity of pronounced variation among individuals within populations represents a paradox that how can such variation exist when selection and drift are constantly acting to remove variation within populations (Mitchell-Olds et al. 2007)? Variation in animal coloration is particularly perplexing because coloration can affect many aspects of an organism including its ability to thermoregulate, to avoid predators, and to attract a mate and/or defend a mate from competitors (Andersson 1994; Ruxton et al. 2004). Hence, animal coloration should be under intense natural and sexual selection. Yet,

theory tells us that natural and sexual selection should reduce genetic variation in coloration, resulting in a single color morph within a population (Lewontin 1974; Bradbury et al. 1987). The maintenance of variation in coloration is even more problematic especially when the color pattern is controlled by a few alleles (Rosenblum et al. 2004; Hoekstra et al. 2006; van't Hof et al. 2011).

There are multiple forms of balancing selection that can, in theory, maintain color polymorphisms. Polymorphisms can be preserved by negative frequency-dependent selection (NFDS),

overdominance, habitat-dependent selection, or trade-offs between different fitness components. Here, we examine the extent to which 2 mechanisms, negative frequency dependence and microhabitat variation in mating success, contribute to the maintenance of 2 discrete color morphs in a freshwater killifish. NFDS has received considerable empirical support in maintaining color polymorphisms (Horth and Travis 2002; Fitzpatrick et al. 2007; Gray and McKinnon 2007; Roulin and Bize 2007; Dijkstra and Border 2018). Negative frequency dependence occurs when rare genotypes have a fitness advantage over common genotypes. An advantage to rare genotypes can involve a number of fitness components including mating success, fecundity, survival, and/or a reduction in predation. In guppies, rare color morphs attract more attention from females and suffer less predation (Olendorf et al. 2006; Bond 2007). In cichlids, sticklebacks, and darters, male aggression is more intensive towards competitors with similar coloration, such that rare male color morphs experience reduced intrasexual competition (Seehausen and Schlüter 2004; Pauers et al. 2008; Dijkstra et al. 2009; Sluijs et al. 2013; Lehtonen 2014; Martin and Mendelson 2016; Moran and Fuller 2018; Tinghitella et al. 2018). In a scale-eating cichlid *Perissodus microlepis*, where animals are curved to either the left or right to pick scales off of other fish, rare morphs have higher foraging rates than common morphs (Hori 1993).

Genetic variation in coloration can also be maintained within populations if there is microhabitat variation such that each color morph can outcompete the others in a particular set of conditions (Hedrick 2006; Dreiss et al. 2012; Burri et al. 2016). The perception of coloration is dependent on lighting environment. Lighting environments are particularly variable in aquatic habitats as the inherent optical properties of water (e.g., materials dissolved or suspended in water) alter the distribution and intensity of the ambient light spectrum (Lythgoe and Partridge 1989). In addition, within any given population, lighting environments differ as a function of time of day and depth. Here, we focus on variation caused by depth. Depth alters the lighting environment due to the absorption of different wavelengths of light (Lythgoe 1988). Studies of cichlids have shown that speciation has occurred along a depth gradient where red color morphs are favored in deeper water and blue color morphs are favored in shallower water (Seehausen et al. 2008). Speciation along depth clines has also been observed in rockfish (*Sebastodes*) (Ingram 2011).

In addition to rarity and microhabitat types, other aspects of male coloration may influence mating success. A variety of pigment types contribute male coloration and their expression levels are influenced by both internal and external factors. Hence, males with identical coloration may still differ in phenotype (Ligon and McCartney 2016). Males of most species possess multiple traits that can signal different aspects of male quality. In terms of coloration, the 3 main pigment types are melanin, carotenoid, and pterin. Red, orange, and yellow ornaments in coloration are primarily composed of carotenoids and pterins (McGraw et al. 2004). In some species, males possess all the 3 types of pigments.

The biology of the melanin pathway is well known. The melanocortin system involves many biological functions such as the immune system, energy homeostasis, and sexual behavior. Hence, selection on melanin might also involve selection on other traits (Ducrest et al. 2008). Despite our depth of knowledge concerning the melanocortin system, the meaning of melanin-based coloration is debated. Some studies indicate that the degree of melanism is quite plastic and is determined by male condition and/or the outcome of past contests (Kekäläinen et al. 2010; Piault et al. 2012; Henschen et al. 2016), while others show that melanism is highly heritable

with small environmental effects (Antoniazza et al. 2010; Roulin and Ducrest 2013; Saino et al. 2013). Therefore, the extent to which melanic traits are honest signals is unclear. Melanic traits might serve as honest badges of status and indicate male aggressiveness, or they might be driven by frequency-dependent selection or local adaption (see Roulin 2016 for a review).

Carotenoid-derived ornaments are assumed to honestly reflect the animals' diet since they cannot synthesize carotenoids *de novo*. Hence, they could truly reflect male foraging ability to potential mates especially when carotenoids are limited in habitats (Olson and Owens 1998; Grether 2000). Moreover, carotenoids are anti-oxidants and benefit the immune system, so carotenoid-derived ornaments are also signals of health (Johnson and Fuller 2015; Megía-Palma et al. 2017).

Pterins have received less attention than melanins or carotenoids. Pterins can be synthesized *de novo* (as can melanin) and have potential immune and antioxidant function (McGraw 2005). However, there are few studies that examine the influence of pterins on male mating success (Johnson and Fuller 2015), and even fewer that consider the effects of all 3 pigment types.

This study focuses on a pronounced color polymorphism present among males in bluefin killifish *Lucania goodei*. In clear spring populations, nearly all males have either solid red or solid yellow anal fins (Fuller 2002). There is no evidence that these color morphs represent different alternative mating strategies. They do not differ in size or in time to sexual maturation, and neither morph acts as a "sneaker" (Fuller 2001, 2002; Johnson and Fuller 2015). Breeding studies have shown that this variation is largely controlled by a single locus where yellow alleles are dominant to red alleles (Fuller and Travis 2004). Yellow and red color morphs are present in all investigated clear water populations (Fuller 2002), which raises the question of how these alleles are maintained within populations in nature. The anal fin can also be blue or a combination of blue and either red or yellow. Males with blue coloration are found primarily in swamps. All males possess the ability to express either red or yellow anal fins, but this coloration is essentially displaced by blue provided that animals have the right genetics and rearing environment (Fuller and Travis 2004). In this study, we focus solely on the red and yellow color morphs and how they are maintained within populations.

A previous study in bluefin killifish showed no evidence of NFDS between yellow and red males. While red males sired more offspring when rare, yellow males did not (Fuller and Johnson 2009). Hence, red males had higher mating success than expected, but yellow males did not. Fuller and Johnson (2009) suggested that a female mating preference was present in the study population. Rare red males benefited from this preference, but common males did not because the preference was, in essence, diluted by the presence of many other red males. Subsequent work has suggested that spring females have a weak preference for red males (Fuller and Noa 2010), but other studies have failed to find such an effect (McGhee et al. 2007; Mitchem et al. in review).

The original test for negative frequency-dependent mating success by Fuller and Johnson (2009) was not perfect. The experiment did not maintain good water quality as algal blooms occurred in some, but not all, of the experimental tanks. This may have altered fish perception of male anal fin morph. In addition, the paternity analysis only allowed assignment of offspring to the rare or common morphs in the tank as a class, rather than to specific fathers and mothers. The latter would have allowed for a more detailed analysis of factors influencing levels of parentage. Fuller and Johnson (2009) only considered 2 additional traits, body length and condition, as

covariates that could potentially explain male mating success. Finally, Fuller and Johnson (2009) did not examine mating success as a function of depth, which may also contribute to the maintenance of the color polymorphism.

In bluefin killifish, the anal fin (red vs. yellow) is controlled by the expression of 2 pterin pigments (likely xanthopterin and drosoterpin) (Johnson and Fuller 2015). Red males express both pterin types, whereas yellow males only express xanthopterin. Throughout this article, we refer to these pigments as yellow (likely xanthopterin) and red pterin (likely drosoterpin). The anal fin also has a melanin black border that predicts the outcome of male/female competition. Males with larger black borders are clearly more dominant (Johnson and Fuller 2015), which is in keeping with predictions based on the biology of the melanocortin pathway (Ducrest et al. 2008). The caudal fin also has reddish/orange coloration, which is due to carotenoid pigments. Previous work in this system indicates that both carotenoid and pterin expression are predictive of health and overall mating success (Johnson and Fuller 2015). Hence, pigment expression (in addition to color morph identity) may be critical in male reproduction.

The goal of this study was to determine whether negative frequency-dependent mating success and/or spatial variation in mating success as a function of depth could account for the maintenance of red and yellow color morphs. Negative frequency-dependent mating success predicts that rare color morphs have a mating advantage over common color morphs. Spatial variation in mating success predicts that each color morph must have a microhabitat where it outperforms the other. In addition to testing these 2 hypotheses, we also asked whether the size, condition, and pigmentation of the male (melanin, carotenoid, and pterin) could account for male mating success.

Materials and Methods

Study system and fish collection

The bluefin killifish is a freshwater fundulid that is native to the southeastern United States of America. Its distribution range is mainly in Florida. During the breeding season (mainly March to mid-summer) (Lee et al. 1980), males protect territories of aquatic vegetation, where spawning and egg attachment occurs. Females can deposit eggs on vegetation throughout the water column ranging from floating vegetation to bottom substrate vegetation (<1.5 m depth) (Fuller 2001). Females spawn their eggs in small batches across multiple males' territories (Fuller and Travis 2001). Both female choice and male-male competition contributes to male mating success (McGhee et al. 2007).

The fish used in this experiment was captured with a seine in May of 2011 from the Upper Bridge population of the Wakulla River, near Tallahassee, Florida. This population is polymorphic in male coloration. Males with blue anal fins are very rare. Both yellow and red males are abundant in this population (Fuller 2002). The fish was transported back to the University of Illinois and housed briefly in a communal oval stock tank (1.85 m in length × 0.86 m in width × 0.65 m in height) before being moved to 12 experimental oval stock tanks (1.85 m in length × 0.86 m in width × 0.65 m in height), which were housed in a glass greenhouse where the temperature was 20°C ~ 30°C and exposed to natural lighting conditions. UV sterilizers were attached to the tanks to prevent algal blooms and maintain water clarity. The following experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois (protocol numbers #11143 and #08183).

Experimental setup

Our first goal was to determine whether negative frequency-dependence or spatial variation in mating success could potentially maintain both color morphs within populations. To do this, we manipulated the ratio of red males to yellow males in each tank. We also stocked tanks with spawning substrates (yarn mops) at 2 depths (surface and bottom) to determine whether red and yellow males differed in spawning location. We created 2 experimental treatments - one where red males were rare (1 red male, 5 yellow males, and 6 females) and another where yellow males were rare (1 yellow male, 5 red males, and 6 females). We performed 7 replicates of each treatment resulting in 14 experimental breeding populations in stock tanks (Supplementary Table 1). In each stock tank, animals could spawn on substrates that were floating on the surface or were on the bottom of the tank (approximately 50 cm deep). For the remainder of this article, we refer to these as "floating mops" and "bottom mops." Hence, the experiment also allowed us to examine whether there is spatial variation in relative fitness.

The yarn mops in each tank were searched at least 3 times a week for eggs. Eggs were removed and maintained in a dilute solution of methylene blue (about 1~2 ppm) to prevent fungal infection until the fry hatched. Fry were fed baby *Artemia* for an additional 3 weeks after hatching. They were then stored in ethanol and frozen until DNA could be extracted using a standard protocol. At the conclusion of the experiment, all the adult fish were euthanized with 0.025% MS-222. For each individual, standard length was recorded using a laminated piece of engineering grid paper (nearest to 1 mm), and wet mass was recorded using an electronic balance (nearest to 0.0001 g).

Our second goal was to determine whether the degree of pigment expression in the anal and caudal fins influenced male reproductive success (whether a male had offspring or not) in *L. goodei*. To do this, we extracted and measured pterins from the anal fin and carotenoids from the caudal fin. We also used photography to measure the amount of black coloration (i.e., melanin) on the anal fin. We examined correlations between the continuous variation in pigmentation and male mating success. At the end of each trial, males were placed against a white background with a color standard, and a digital picture was taken of the left side of each male using a Nikon D3300 camera. A Camera PictoColor 4.5 Photoshop plug-in was subsequently used to standardize the light and color levels of each picture. The caudal and anal fin were removed and spread out on a glass slide. Rough measurements to the nearest 1 mm of each fin were taken by treating the fin as a parallelogram and noting the length of its proximal and distal ends and the distance between the 2. The fins were stored at -80°C until pigment could be quantified. The caudal peduncles of all adults were removed and stored in ethanol at -80°C until DNA was extracted.

Parentage analysis

Parents and offspring were typed at the following 3 highly polymorphic microsatellite loci: CA (Fuller and Johnson 2009), AC17 (Burg et al. 2002), and Lg1 (Creer and Trexler 2006). Forward primers were labeled with VIC (CA), 6FAM (AC17), or Pet (Lg1). The loci were amplified in 1 multiplex reaction according to the standard protocol in the QIAGEN Multiplex Polymerase chain reaction (PCR) Kit. The PCR products were run on an ABI Prism 3730xl Analyzer at the University of Illinois' W.M. Keck Center for Comparative and Functional Genomics. Fragment sizes were scored using GeneMapper software (Applied Biosystems) and verified manually. We then used CERVUS V 3.0.3 (fieldgenetics.com) to assign parentage to the fry

(Kalinowski et al. 2007). Each stock tank was analyzed separately, and offsprings were assigned parentage based on 80% likelihood. Only a small number of offspring (38 of 1051) failed to have parentage assigned to them, due to either unresolvable parentage or poor DNA quality.

Many replicates experienced adult mortality. These individuals were included in the CERVUS parentage simulations as un-sampled potential parents. With the exception of 1 deceased female, dead females did not contribute any offspring, and we treated the replicate as having been formed without them. However, 3 deceased males did leave a notable number of offspring. We were able to reconstruct his or her genotype, which helped further identify parentage, and deduce the color morph of the missing males by examining the body and/or deducing it from the other morphs in the tank. However, we were unable to measure pigmentation, and our sample sizes reflect this. In other cases, individuals with pale fin coloration were initially misidentified as the wrong sex or morph. This altered our gender and morph ratios (Supplementary Table 1), but it did not affect our ability to detect the effect of pigmentation on paternity, and in fact more accurately represents the pigment variation found in nature.

Coloration analysis

The methods here follow Johnson and Fuller (2015), where the pigments were extracted and identified. Briefly, to quantify pterins and carotenoids, we used 2 solvents, 1% NH₄OH and 1: 1 mixture of hexane: tert-butyl methyl ether, to extract these 2 pigments from fins and partition carotenoids from pterins. This method of identifying pterins and carotenoids has been widely applied to coloration studies in different animals (Kikuchi et al. 2014; Steffen et al. 2015; Cuervo et al. 2016). Individual anal and caudal fins were thoroughly ground with a mortar and pestle in 1% NH₄OH, and then a 1: 1 mixture of hexane: tert-butyl methyl ether was added when eluting carotenoids. The absorption spectra of these 2 solvent layers were examined to determine pigment class. While eumelanin and structural coloration did not go into solution, pterins could be identified by a strong UV absorption in the NH₄OH layer (Hill and McGraw 2006). Carotenoids were identified by a characteristic pattern of absorbance in the hexane: tert-butyl methyl ether solvent (McGraw 2005).

The caudal fins were homogenized in 1 ml 1% NH₄OH. The ground material and solvent were transferred to a fresh tube and an equivalent volume of a 1: 1 hexane: tert-butyl methyl ether solvent was added. The solution was vortexed, centrifuged, and the 2 solvents were separated. Carotenoids were present in the top layer, the hexane: tert-butyl methyl ether layer. The absorption of the hexane: tert-butyl methyl ether layer was measured on a spectrophotometer, and the height of the absorption peak at 445 nm was used to quantify carotenoid levels (Johnson and Fuller 2015).

For measurements of pterins in anal fins, the anal fins were homogenized in 400 μL 1% NH₄OH, centrifuged, and the resulting supernatant was collected. The height of the absorption peak at 398 nm was used to quantify yellow pterin pigment (xanthopterin) and that at 498 nm was used to quantify red pterin pigment (drosophterin) (Johnson and Fuller 2015). Total anal fin pterin was measured as red and yellow pterin (absorption) summed. Yellow males express only the yellow pterin. Red males express both the yellow and red pterin.

Anal fin melanin could not be analyzed using absorption spectroscopy, so digital picture analysis in ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, imagej.nih.gov/ij/) was used instead. Small differences in magnification between

pictures were corrected for by scaling each image to a size standard. The anal fin was isolated using the freehand selection tool, and the image was converted to black and white using the adjust threshold function and selecting black and white threshold color. The image was then converted to a binary image, and the area of the black band was calculated with the measurement tool.

Statistical analysis

We first report basic statistics on paternity, the skew in reproduction in males and females, and general associations between size, condition, and mating success. We measured reproductive skew (S) separately for males and females in each replicate using the formula presented in Keller (1993) that results in a value from 0 (no skew) to 1:

$$S = \frac{\nu \cdot N_b + N_n}{N_b + N_n}$$

where, N_b is the number of adults that bred at least 1 offspring, N_n is the number of individuals assigned 0 offspring, and ν is the standard deviation among breeders that have at least 1 offspring in the proportion of total offspring assigned parentage (Supplementary Table 1). Reproductive skew was measured for males and females in each tank (2 genders × 14 tanks = 28 values total). We then tested for differences in reproductive skew between genders using analysis of variance (ANOVA) and also for differences in male reproductive skew between our treatments (red rare/yellow common vs. yellow rare/red common, Supplementary Table 1).

We first asked whether negative frequency dependence in mating success could potentially maintain both color morphs within populations. Negative frequency-dependent mating success predicts that rare males have a mating advantage over common males. For each male, we calculated the total mating success (% of total offspring sired by a male), the mating success on floating mops (% of offspring from floating mops sired by a male), and the mating success on bottom mops (% of offspring from bottom mops sired by a male). We then calculated the average mating success for yellow and red males for each tank. We used linear models to determine whether the average male mating success of red and yellow males varied depending on male color morph (red vs. yellow), rarity status (rare vs. common), and the interaction between the male color morph and rarity status for each of the 3 measures of male mating success (% total offspring, % offspring from floating mops, and % offspring from bottom mops). To do this, we used the “lmer” function in R (lme4 package). The experimental tank was treated as a random effect in all 3 models. We used a Type 3 analysis in the “car” package to determine the effects of each term.

We next asked whether males varied in where they spawned their offspring. Here, we measured the number of offspring that males sired on bottom mops relative to the number that they sired on floating mops. This analysis used individual males as the level of observation and, by default, excluded males that did not sire any offspring. We asked whether male color morph (red vs. yellow), rarity (rare vs. common), and the interaction between rarity and color morphs affected where males spawned their offspring. Experimental tank was a random effect. To do this, we used binomial model in R using the “glmer” function from the “lme4” package. We used a Type 3 analysis in the “car” package to determine the effects of each term. Our initial model suffered from over-dispersion, so we included individual ID as an additional random effect (Harrison 2014).

Finally, we examined the effect of male size, condition, and pigments levels on male mating success. We first examined Pearson correlations between standard length, condition, anal fin size, caudal

fin size, pigmentation (melanin, red pterin, yellow pterin, and total pterin carotenoid levels), and the 3 measures of male mating success (% total offspring, % offspring from floating mops, and % offspring from bottom mops). The condition of each fish was calculated as the residuals of the \log_{10} of weight regressed on the \log_{10} of standard length (Bolger and Connolly 1989).

We then asked whether inclusion of these traits altered our interpretation of our experimental treatments. Because many of the characters were significantly correlated (see results), we used principal components analysis to obtain composite scores of 6 male characters (standard length, condition, caudal fin size, yellow pterin, carotenoid, and melanin). We excluded red pterin, total pterin, and anal fin size from the analysis because these traits varied between yellow and red males. We examined the results of the principal components analysis and retained the first 3 principal components. We then performed 4 analyses. The first analysis simply asked whether male color morph, rarity, the interaction between rarity and color morph, and the first 3 principal components explained whether or not males mated, which we refer to as mating status. For this analysis, we categorized males as either having mated or not. We used a binomial model in R using the “glmer” function from the “lme4” package. We then performed another 3 analyses where we examined the effects of male color morph, rarity, their interaction, the first 3 principal components on total male mating success (% of total offspring sired), male mating success on floating mops (% of offspring from floating mops sired), and male mating success on bottom mops (% of offspring from bottom mops sired). Here, we used a linear model using the “lmer” function from the “lme4” package. For all 4 models, experimental tank was treated as a random effect. Type 3 models were used throughout.

The raw data for this experiment have been deposited in Dryad (number to be entered upon acceptance).

Results

Testing for negative frequency-dependent mating success

We identified parentage in a large number of fry (Supplementary Table 1). In total, 1,560 eggs were collected across the experiment. From those eggs, 1,060 fry hatched and survived long enough to have DNA extracted. A subset of those (1,051) were typed, and of those, 1,011 (96%) were successfully assigned parentage by CERVUS at 80% confidence level or above. Reproductive skew did not differ between males and females (paired t-test on male-female skew across the 14 tanks: $t_{13} = 0.951$, $P = 0.359$), nor did male reproductive skew vary between tanks in which red or yellow males were rare ($F_{1, 12} = 3.01$, $P = 0.1083$) (Supplementary Table 1). There was no difference between treatments in the number of eggs laid in the tanks after correcting for experimental duration and the number of females in the tanks ($F_{1, 12} = 2.48$, $P = 0.1434$).

There was no evidence for negative frequency-dependent mating success when considering all of the data. Rarity status had a marginally significant effect on total male mating success (Table 1A, $P = 0.043$) but common males had slightly higher mating success than rare males (Figure 1A). This effect was present for males of both color morphs (average mating success: common-yellow = 0.14, rare-yellow = 0.07, common-red = 0.18, rare-red = 0.13). Removal of a large outlier rendered the pattern even more significant (rarity: $F_{1, 11} = 63.8$, $P < 0.0001$) with common males having higher mating success than rare males. There was little evidence that mating success differed due to male coloration or due to the interaction

Table 1. Mating success (proportion of offspring sired) as a function of male color morph, rarity, and their interaction

A: Total mating success (proportion of offspring sired)			
Term	F	DF (num, denom)	P
(Intercept)	66.8	1, 12	<0.0001
Color	0.83	1, 12	0.3805
Rarity	5.1	1, 12	0.0433
Color × Rarity	0.64	1, 12	0.4408
B: Mating success on floating mops			
Term	F	DF (num, denom)	P
(Intercept)	38.1	1, 12	<0.0001
Color	0.1	1, 12	0.7239
Rarity	6.8	1, 12	0.0225
Color × Rarity	0.1	1, 12	0.7378
C: Mating success on bottom mops			
Term	F	DF (num, denom)	P
(Intercept)	78.7	1, 12	<0.0001
Color	7.7	1, 12	0.0169
Rarity	4.4	1, 12	0.0584
Color × Rarity	4.0	1, 12	0.0674

The analysis considers the tank means of mating success for red and yellow males (and their associated rarity status) across the 14 tanks. Tank is treated as a random effect. “num” refers to numerator, and “denom” refers to denominator. Terms with $P < 0.05$ in bold. $P < 0.10$ but $P > 0.05$ in italics.

between male coloration and rarity. We found nearly identical results for mating success on floating mops (Figure 1B, Table 1B). This was not surprising as 84% of the offspring came from floating mops. A significant effect of rarity was present ($P = 0.0225$), where common males had higher mating success than rare males, and the pattern became much stronger after the removal of a large outlier (rarity: $F_{1, 11} = 111.1$, $P < 0.0001$). The effect of rarity had a similar effect on males of both color morphs (average mating success on floating mops: common-yellow = 0.18, rare-yellow = 0.06, common-red = 0.18, rare-red = 0.09). Removal of this data point also resulted in a marginally significant ($F_{1, 11} = 4.5$, $P = 0.0575$) of color where yellow color morphs had slightly higher mating success on floating mops.

A different pattern emerged from bottom mops. Rare males had slightly higher mating success than common males (Table 1C, Figure 1C, $P = 0.058$). This was particularly so for red males. There was a statistically significant affect male coloration ($P = 0.0169$), where red males had higher mating success than yellow males. A marginally significant interaction was also present, where red males were more likely to have high mating success on bottom mops when they were rare ($P = 0.0674$, average mating success: common-yellow = 0.13, rare-yellow = 0.14, common-red = 0.17, and rare-red = 0.35). Red males had 2X greater mating success on bottom mops when rare than when they were common, and >2X greater mating success on bottom mops than either common-yellow or rare-yellow males.

Testing for differences in spawning location due to depth

Here, we asked whether color morph and rarity affected where males spawned. Eleven of 87 males in the experiment did not successfully reproduce, so they were excluded from the analysis. The results largely matched the patterns found for male mating success on bottom mops. Rarity influenced where males spawned (Table 2, Figure 2, $P = 0.0011$). Rare males spawned more of their

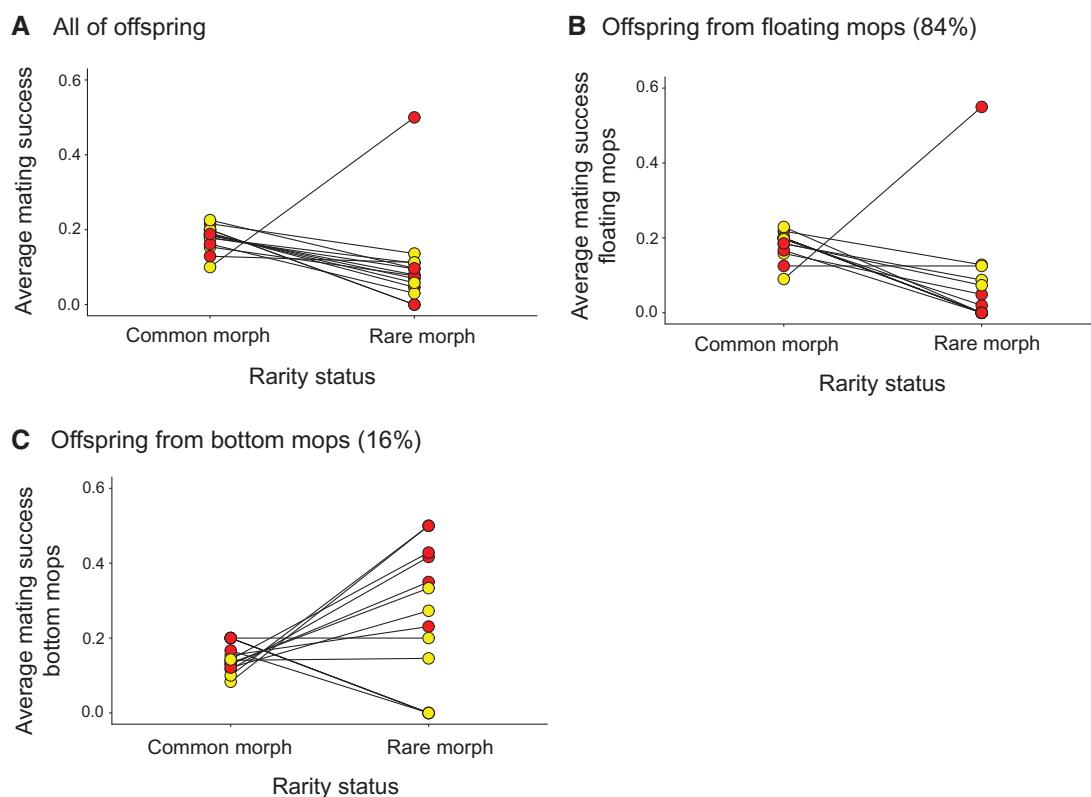


Figure 1. The average mating success of red and yellow males as a function of rarity. Lines denote averages of red and yellow males from the same experimental tank. Red fill denotes red males. Yellow fill denotes yellow males. (A) Average mating success (percentage of the total offspring sired for a tank). (B) Average mating success on floating mops (percentage of the offspring sired from floating mops). (C) Average mating success on bottom mops (percentage of the offspring sired from bottom mops). Note that 84% of all offspring were spawned on floating mops and 16% were spawned on bottom mops.

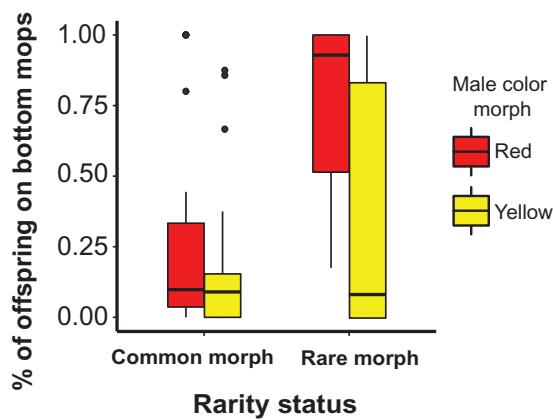


Figure 2. Male spatial distribution of offspring as a function of rarity status (common vs. rare) and color morph (red vs. yellow). The y-axis shows the proportion of offspring on the bottom mop versus the total of offspring individual males. $N=76$. Eleven males were excluded because they did not sire any offspring.

offspring on the bottom mops than on the floating mops relative to common males. There was also a significant effect of male coloration, where red males spawned more of their offspring on bottom mops than on floating mops relative to yellow males ($P=0.035$). Finally, there was a marginally significant interaction due to the fact that the rare-red males placed more offspring on the bottom mops than common-red, common-yellow, and rare-yellow males (Figure 2, Table 2).

Male phenotypical characters and reproductive success

Here, we asked whether red and yellow males differed in other traits that might affect male mating success. Three males with incomplete data were excluded from this analysis, leaving us with 84 males. In addition, a large outlier was also present among the carotenoid data, which was excluded for analyses of carotenoid levels.

Males in each tank were assigned a color morph (red/yellow by AJ). Visual assignment matched the absorption spectroscopy data from the anal fins. Red and yellow males did not differ in standard length ($F_{1, 82} = 1.59, P = 0.211$), condition ($F_{1, 82} = 0.16, P = 0.691$), or caudal fin size ($F_{1, 82} = 0.01, P = 0.936$), but red males did have larger anal fins than yellow males ($F_{1, 82} = 12.12, P = 0.001$). We calculated the residuals of a regression of anal fin size on standard length to determine whether this was a genuine pattern or simply an effect due to subtle differences in size. The analysis revealed that the residuals were larger for red males than yellow males ($F_{1, 82} = 16.67, P = 0.0001$), indicating that red males have larger anal fins regardless of body size. Red and yellow males did not differ in the amount of carotenoid ($F_{1, 81} = 1.30, P = 0.258$), melanin ($F_{1, 82} = 0.97, P = 0.327$), or yellow pterin ($F_{1, 82} = 0.15, P = 0.696$). However, not surprisingly, red males had significantly more red pterin ($F_{1, 82} = 64.10, P < 0.0001$) and more total pterin ($F_{1, 82} = 5.39, P = 0.023$) than yellow males.

We next asked whether there were correlations between continuously varying traits: standard length, condition, anal fin size, caudal fin size, carotenoid, yellow pterin, red pterin, total pterin, melanin, the proportion of total offspring sired, the proportion of offspring sired from floating mops, and the proportion of

Table 2. General linearized model examining the proportion of eggs laid on bottom mops versus floating mops by individual males

Term	χ^2	df	P
Intercept	9.02	1	0.0027
Color	4.45	1	0.0348
Rarity	10.66	1	0.0011
Color × Rarity	3.01	1	0.0827

The model assumes a binomial distribution with a logit link function. Tank and individual identity are treated as random effects. Terms with $P < 0.05$ in bold. $P < 0.10$ but $P > 0.05$ in italics. $N = 76$. Eleven males (out of 87 total) did not sire any offspring and were excluded from the analysis.

offspring sired from bottom mops. Our analysis revealed that there were several statistically significant correlations among these variables. The 3 pigment classes (carotenoid, pterin, and melanin) were loosely correlated with one another. Melanin was correlated with yellow pterin, red pterin, and total pterin (Supplementary Figure 1A–C). Carotenoid was correlated with yellow pterin and total pterin (Supplementary Figure 2A–B). Red and yellow pterin were correlated with one another (Supplementary Figure 2C) and with total pterin. All 3 pigment classes were loosely correlated with male mating success (melanin: Supplementary Figure 3A–C; carotenoid: Supplementary Figure 4A–C; yellow pterin: Supplementary Figure 5A–C; red pterin: Supplementary Figure 6A–C). Carotenoid was loosely correlated with the proportion of total offspring sired and the proportion of offspring sired on floating mops. Both yellow pterin and melanin were correlated with the proportion of total offspring sired, the proportion of offspring sired from floating mops, and the proportion of offspring sired from bottom mops. Red pterin was loosely correlated with the proportion of total offspring sired and strongly correlated with the proportion of offspring sired on bottom mops. This result is in keeping with the result that red males have higher mating success on bottom mops than do yellow males.

We next asked whether incorporation of pigment levels, standard length, condition, and fin sizes altered the results of our treatments. We used a principal components analysis to summarize the broad patterns of covariation among these traits and then asked whether or not inclusion of the principal component scores dramatically altered our treatment effects. We included standard length, condition, caudal fin size, carotenoid, yellow pterin, and melanin values in the principal components analysis. Red pterin and anal fin size were excluded because they differed between yellow and red males. Supplementary Table 3 shows the result of the principal components analysis. The first 3 principal components accounted for over 50% of the variation in the traits. PC1 loaded strongly onto all traits except standard length. PC2 loaded strongly onto standard length, caudal fin size, and carotenoid but negatively onto yellow pterin and melanin. PC3 loaded strongly onto condition and caudal fin size, but negatively onto standard length, yellow pterin, and melanin.

Table 4 shows the results of our analyses. PC1 had a strong effect on whether or not males mated (Table 4A, Figure 3A). Males that failed to mate had low PC1 values. Not surprisingly, PC1 also affected total male mating success (Table 4B, Figure 3B). This analysis was similar to the previous analysis on tank means (Table 1A). Here, there was a marginal effect of rarity ($P = 0.071$) where common males had higher mating success than rare males. The same patterns were seen

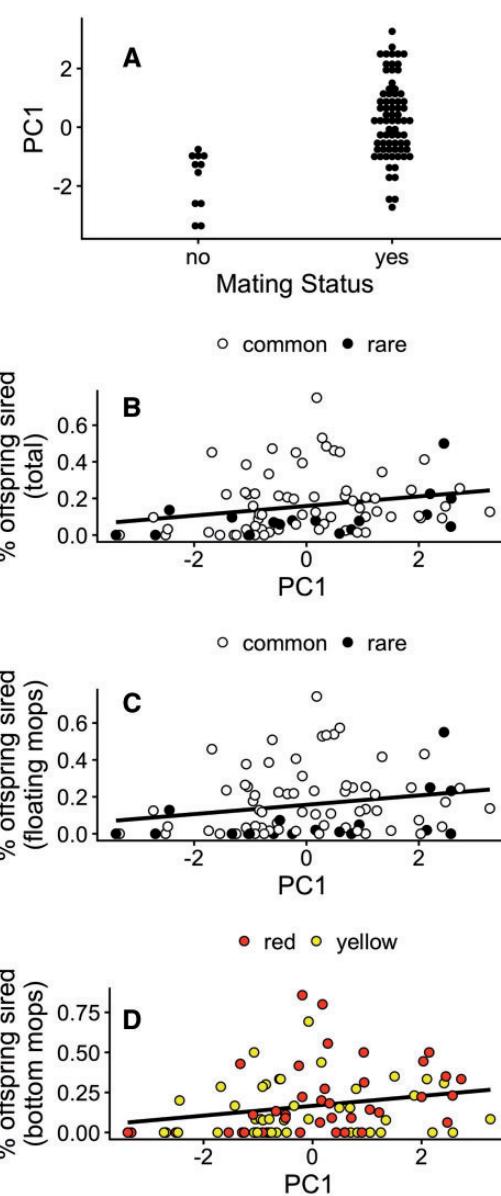


Figure 3. (A) The relationship between PC1 and whether or not a male spawned any offspring. (B–D) The relationship between PC1 and (B) the proportion of total offspring sired, (C) the proportion of offspring sired on floating mops, and (D) the proportion of offspring sired on bottom mops. $N = 84$ for all three graphs. Graphs B and C indicate whether males were common (open circles) or rare (dark circles). Graph D indicates whether males were red or yellow color morphs.

for male mating success on floating mops. Higher levels of PC1 were loosely associated with increased mating success (Table 4C, Figure 3C, $P = 0.0528$), and common males had an advantage over rare males ($P = 0.0235$). Finally, the analysis of male mating success on bottom mops again indicated that red males had an advantage over yellow males (Table 4C, Figure 3D, $P = 0.0258$). The interaction between color and rarity was marginally significant ($P = 0.0678$) due to the fact that red males had higher mating success when rare (mating success on bottom mops: rare-red = 0.347, common-red = 0.169, rare-yellow = 0.124, common-yellow = 0.139). There were also marginal effects of PC1 where higher levels of PC1 were loosely associated with increases in mating success on bottom mops.

Table 3. Pearson's correlation coefficients (above the diagonal) and P-values (below the diagonal).

	SL	Condition	Anal Fin Area	Caudal fin area	Carot	Yellow pterin	Red pterin	Total pterin	Mel	% offspring (total)	% offspring (bottom)	% offspring (top)
SL		0.008	0.269	0.090	0.291	0.110	-0.059	0.070	-0.018	0.073	-0.067	0.109
Condition	0.943		0.144	0.349	0.141	0.379	0.171	0.361	0.336	0.090	0.121	0.081
Anal fin area	0.013	0.193		-0.157	0.035	0.210	0.339	0.279	0.122	0.078	0.065	0.070
Caudal fin area	0.416	0.001	0.153		0.313	0.216	0.106	0.208	0.140	0.064	0.086	0.051
Carotenoid	0.008	0.204	0.754	0.004		0.324	0.003	0.261	0.174	0.229	0.119	0.224
Yellow pterin	0.319	0.000	0.055	0.049	0.003		0.471	0.959	0.590	0.267	0.222	0.235
Red pterin	0.596	0.119	0.002	0.339	0.975	0.000		0.702	0.275	0.221	0.333	0.160
Total pterin	0.528	0.001	0.010	0.057	0.017	0.000	0.000		0.564	0.287	0.286	0.242
Mel	0.874	0.002	0.269	0.205	0.115	0.000	0.011	0.000		0.219	0.215	0.182
Percentage offspring (total)	0.507	0.415	0.481	0.564	0.038	0.014	0.043	0.008	0.045		0.520	0.978
Percentage offspring (bottom)	0.543	0.271	0.559	0.438	0.285	0.043	0.002	0.008	0.050	0.000		0.346
Percentage offspring (top)	0.322	0.461	0.529	0.642	0.042	0.031	0.146	0.027	0.097	0.000		0.001

N=84, except for correlations involving carotenoid where a single, large outlier was removed. Carot=carotenoid, mel=melanin. Values in bold denote $P < 0.05$.

Discussion

Mechanisms maintaining variation

By manipulating the ratios of red and yellow morphs in bluefin killifish, we were able to test whether rare morph males have a mating advantage that results in increased paternity. We show here, in corroboration with previous results (Fuller and Johnson 2009), that rare males have no overall mating advantage. In fact, rare-morph males actually sired significantly fewer offspring than common-morph ones in our experimental setup, particularly on the floating mops. Theoretically, our results could have been affected by mortality in eggs or fry that prevented us from assigning parentage to 100% of the offspring. However, this scenario would require that the offspring of rare color morphs suffer higher mortality than the offspring of common color morphs, and this is extremely unlikely. We can, therefore, be reasonably certain that negative frequency-dependent sexual selection is not operating to maintain the red/yellow anal fin polymorphism in bluefin killifish.

These findings are in keeping with previous work. Fuller and Johnson (2009) performed a similar experiment testing for negative frequency-dependent mating success between yellow and red males. They found that red males (but not yellow males) had a mating advantage when rare. The current experiment produced a similar pattern in that red males had an advantage when rare, but only on the bottom mops. The 2 studies are also similar in that yellow males never had a mating advantage when rare. The explanation proposed by Fuller and Johnson (2009) was that a female mating preference was present in this spring population favoring red males. Subsequent work by Fuller and Noa (2010) showed that, indeed, there is a slight mating preference for red males over yellow and blue males for spring females. The finding that red males have an advantage when rare suggests that red males receive a disproportionate share of matings with females when rare, but that this advantage is diluted when females have many red males to choose among. Fuller and Johnson (2009) also did not maintain crystal clear water, which might have made it easier for females to exert mating preferences without being disrupted by competing males. The bottoms of our stock tanks had more nooks and crannies where animals are less visible to competing males. Hence, bottom substrates may have

possibly provided an area where females can exert preference without being disrupted by competing males. Common males had higher mating success than rare males on floating mops (Figure 1B), but rare males had higher mating success than common males on bottom mops (Figure 1C). In addition, more eggs were obtained from floating mops than from bottom mops. Why this occurs is unclear? One possibility is that fish prefer to place their eggs on floating mops and that common males compete intensely over these substrates. However, Sandkam and Fuller (2011) asserted that bluefin killifish have no clear preference for spawning on either floating mops or bottom ones. However, in their experiment, there was only 1 male and 1 female in each trial. Therefore, male-male competition for spawning substrates was precluded. However, there were multiple males in an experimental tank in our study. Hence, the spawning substrates could have become limited when males competed to establishing their own territories. The spatial difference in mating success between common and rare males might imply that bluefin killifish prefer floating mops to bottom ones and display stronger male aggression towards opposite-morph. Such a scenario has been shown in a cichlid, *Astatotilapia burtoni*, and in the white-throated sparrow (Korzan and Fernald 2006; Horton et al. 2012).

An alternative hypothesis is that spatial variation in mating success allows for the maintenance of the 2 color morphs. Here, we did find that on bottom mops, red males had higher mating success on bottom mops and that this was particularly so when they were rare (Figure 1C, Table 3). This finding is in keeping with Fuller and Johnson (2009) who found that red males had increased mating success when rare, but that there was no reciprocal effect for yellow males (Figure 1C). Likewise, here, there was no evidence that yellow males had increased mating success on floating mops (Figure 1B) nor that they had heightened mating success when rare (Figure 1A). In order for negative frequency dependence to maintain the variation in coloration, both morphs must have increased fitness when rare. This was clearly not the case. Likewise, in order for spatial variation in mating success to maintain the variation, each color morph must have a microhabitat where it outperforms the other. While red had higher mating success than yellow males on bottom mops, the reverse was not true for yellow males. Yellow males did not outperform red males on floating mops.

Table 4. Type 3 analyses on the effects of male color, rarity, male color \times rarity, PC1, PC2, and PC3 on (A), (B), (C) and (D)

A: Mating status (yes or no)			
Term	X ²	DF	P
(Intercept)	10.8812	1	0.0010
Color	0.7557	1	0.3847
Rarity	0.0015	1	0.9691
Color \times Rarity	2.3678	1	0.1239
PC1	9.4215	1	0.0021
PC2	0.2651	1	0.6067
PC3	0.2983	1	0.5849
B: Total mating success (proportion of offspring sired) ^a			
Term	F	DF (num, denom)	P
(Intercept)	40.3449	1, 23.8	<0.0001
Color	0.3998	1, 70.2	0.5292
Rarity	3.366	1, 69.1	0.0709
Color \times Rarity	0.2456	1, 24.8	0.6246
PC1	4.8099	1, 58.5	0.0323
PC2	0.0191	1, 63.8	0.8907
PC3	2.0448	1, 52.6	0.1587
C: Mating success on floating mops ^b			
Term	F	DF (num, denom)	P
(Intercept)	27.5609	1, 23.8	<0.0001
Color	0.0482	1, 70.2	0.8269
Rarity	5.3638	1, 69.1	0.0235
Color \times Rarity	0.0103	1, 24.8	0.9201
PC1	3.9061	1, 58.5	0.0528
PC2	0.0354	1, 63.8	0.8515
PC3	2.1978	1, 52.6	0.1442
D: Mating success on bottom mops ^c			
Term	F	DF (num, denom)	P
(Intercept)	55.125	1, 23.8	0.0000
Color	5.1907	1, 70.2	0.0258
Rarity	2.3244	1, 69.1	0.1319
Color \times Rarity	3.6466	1, 24.8	0.0678
PC1	3.3457	1, 58.5	0.0725
PC2	1.2204	1, 63.8	0.2734
PC3	0.0603	1, 52.6	0.8070

N=84 for all 4 tables. **Table 4A** shows a generalized linear model that assumes a binomial distribution with a logit link function. Analyses 4B–4D are linear models of proportional data. All analyses include tank as a random effect. DF=degrees of freedom. num = numerator, denom = denominator. Terms with P<0.05 in bold. P<0.10 but P>0.05 in italics. N=76., ^aPercentage of total offspring spawned., ^bPercentage of offspring spawned on floating mops., ^cPercentage of offspring spawned on bottom mops.

While unable to explain the maintenance of genetic color morphs, our study does shed light on some of the determinants of male mating success. Our results suggest that microhabitat variation in light quality affects the mating success of the red color morph. Our previous research suggests that bluefin killifish have preferences for colors that contrast with available light (Fuller et al. 2010; Fuller and Noa 2010; Johnson et al. 2013). In clear spring water, (like that of our source population and which our stock tanks mimicked), longer wavelengths are attenuated more quickly than shorter wavelengths. Thus, there are relatively fewer red wavelengths in spawning substrates at the bottom of the water column than at the top. Of course, this would indicate that red males should be found more often at depth, which is not readily apparent in the wild (Fuller 2001). Nonetheless, this suggests a potentially fruitful avenue of future research.

Another potential explanation for why rare-red males sired more offspring on the bottom mops is that rare-red males simply spent more time at the bottom. This might occur if common-yellow males expelled the rare-red males from floating mops or if rare-red males actively chose to stay at bottom. These 2 reasons are not mutually exclusive. The different offspring spatial composition between common-red and rare-red males suggests a potentially fruitful avenue of future research on the color morph spatial distribution along the water depth in the wild populations, and more importantly points out that environmental heterogeneity may assist in maintaining the color polymorphism of bluefin killifish.

The pattern in bluefin killifish stands in contrast to the guppy *Poecilia reticulata*, where NFDS occurs through at least 2 known mechanisms (mating and predation) (Hughes et al. 1999; Olendorf et al. 2006). The source of this disparity might stem from the different effective population sizes of the 2 species. Guppy populations are small and can become quite isolated, especially during the dry season (Griffiths and Magurran 1997). In addition, numerous studies have demonstrated that guppies can suffer from inbreeding depression (Mariette et al. 2006; Pitcher et al. 2008; Johnson et al. 2010). These factors may favor behaviors that facilitate inbreeding avoidance, such as a preference for rare males. In contrast, bluefin killifish have extremely large population sizes (Turner et al. 1999), and females actively allocate their eggs across multiple males (Fuller 2001), which lessens the potential consequences of inbreeding. Without the potential for inbreeding depression, female bluefin killifish might not benefit from avoiding mating with common-morphs. On the contrary, if rare males are rare because they have low fitness, then preference for rare males might be maladaptive.

Effects of male phenotypical characters

Males with high levels of pigmentation (yellow pterin, red pterin, total pterin, melanin, and carotenoid) were more likely to sire offspring. All 3 types of pigmentation (melanin, pterin, carotenoid) were strongly associated with whether or not a male had offspring (**Table 3**, **Figure 3**) and were correlated with mating success. Our previous work looked at the effect of pigmentation on dominance and found a strong effect of anal fin melanin on dominance, and thus access to females (Johnson and Fuller 2015). The results of the current study confirm those results and suggest that dominance in this species can directly translate into increased likelihood of mating, even when females can presumably avoid aggressive males by hiding or preferentially mating with other males. Males with high levels of carotenoids and pterins were also more likely to sire offspring. Exactly what these 2 pigments are signaling is unclear. We argued previously that higher levels of carotenoids and pterins may be attractive to females because these pigments signal condition in the case of carotenoid, and parasite load in the case of both pterin and carotenoid abundance (Johnson and Fuller 2015). In this study, condition was significantly correlated with pterin and melanin, but the correlation between carotenoid and condition was not statistically significant (**Table 3**). Possible reasons for these discrepancies include the fact that the fish used in this experiment were smaller, younger, and more heavily parasitized than the fish used in Johnson and Fuller (2015). Hence, the value of pigmentation as a signal of health and condition may vary due to natural conditions.

In conclusion, this study found little evidence that negative frequency-dependent mating success can account for the maintenance of the yellow-red genetic color polymorphism in bluefin killifish. Red males did have a mating advantage on bottom spawning substrates when they were rare, but yellow did not have a reciprocal

advantage. Hence, negative frequency-dependent mating success cannot account for the maintenance of the 2 color morphs. Red males may benefit from a female mating preference when they are rare, particularly when spawning on bottom substrates. This observation does suggest that variation in the lighting environment might alter either the attractiveness of the males or the ability of females to exert mating preferences.

The question remains as to what maintains such striking levels of polymorphism across multiple populations. Negative frequency-dependent fitness could emerge from other selective forces such as predation. Another possibility is that balancing selection is present in the form of overdominance (heterozygote advantage). Transcriptomes and a linkage map for bluefin killifish have been published (Kozak et al. 2014; Berdan et al. 2018). Fuller is currently assembling the bluefin killifish genome. Hence, it may be feasible to identify the red/yellow locus and test for overdominance. This study did find that the mating success of red males is heightened on deeper spawning substrates, particularly when they were rare, and also found positive correlations between overall pigmentation and reproductive success, reaffirming the importance of coloration on mating dynamics in this species.

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Author contributions

The experiment was designed by A.M.J. and R.C.F., and carried out by A.M.J. The data were collected by A.M.J. and analyzed by C-H.C. The manuscript was prepared by C-H.C. and supervised by R.C.F.

Conflict of interests

The authors report no conflict of interests.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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