**Title.** Consistent female preference for rare and unfamiliar male color patterns in wild guppy populations.

**Short Title.** Rare male effect in natural guppy populations.

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**Data archiving:** Analyses reported in this article can be reproduced using the data provided by Valvo et al. (2019).

**Abstract.** How genetic variation is maintained in ecologically important traits is a central question in evolutionary biology. Male Trinidadian guppies, *Poecilia reticulata*, exhibit high genetic diversity in color patterns within populations, and field and laboratory studies implicate negative frequency-dependent selection in maintaining this variation. However, behavioral and ecological processes that mediate this selection in natural populations are poorly understood. We evaluated female mate preference in 11 natural guppy populations, including paired populations from high-and low-predation habitats, to determine if this behavior is responsible for negative frequency-dependent selection and to evaluate its prevalence in nature. Females directed significantly more attention to males with rare and unfamiliar color patterns than to males with common patterns. Female attention also increased with the area of male orange coloration, but this preference was independent of the preference for rare and unfamiliar patterns. We also found an overall effect of predation regime; females from high-predation populations directed more attention toward males than those from low-predation populations. Again, however, the habitat-linked preference was statistically independent from the preference for rare and unfamiliar patterns. Because previous research indicates that female attention to males predicts male mating success, we conclude that the prevalence of female preference for males with rare and unfamiliar color patterns across many natural populations supports the hypothesis that female preference is an important process underlying the maintenance of high genetic variation in guppy color patterns.

**Key Words.** Frequency dependent selection, female mate preference, maintenance of genetic variation, rare male effect, color pattern polymorphism

## Introduction

Genetic variation in ecologically-important traits is a pre-requisite for evolutionary change; understanding processes that maintain this variation has fundamental implications for conservation biology, agriculture, and medicine. Such variation is ubiquitous, yet its maintenance in the face of natural selection is poorly understood (Mitchell-Olds et al. 2007, Leffler et al. 2012, Charlesworth 2015). Mutation, gene flow, and various kinds of “balancing” natural selection can promote the maintenance of polymorphism, but we lack a general understanding of which of these processes is most important in nature (Barton and Turelli 1989, Mitchell-Olds et al. 2007, Delph and Kelly 2014, Troth et al. 2018).

Negative frequency-dependent selection (NFDS) is a type of balancing selection where rare genotypes experience a fitness advantage. Theory suggests that NFDS is capable of  maintaining high genetic and phenotypic variation (Ayala and Campbell 1974) and this process has been implicated in some well-known examples of polymorphism for ecologically important traits (e.g. Cain and Sheppard 1950; Shuster and Wade 1991; Sinervo and Lively 1996; Hugie and Lank 1997; Gigord et al. 2001;

Bleay et al. 2007; Takahashi et al. 2010; Mokkonen et al. 2011; Rivkin et al. 2015; Indermaur et al. 2018). A well-supported example of NFDS is found in Trinidad guppies (*Poecilia reticulata*), which exhibit high levels of within-population variation in male color patterns (Figure 1). Conspicuous coloration is male-limited in guppies, and color pattern variation is highly heritable (Houde 1997, Brooks and Endler 2001, Hughes et al. 2005). Both natural and sexual selection appear to favor males with rare or unfamiliar color patterns in this species (Farr 1977, Hughes et al. 1999, 2013, Eakley and Houde 2004, Olendorf et al. 2006, Zajitschek and Brooks 2008, Hampton et al. 2009, Graber et al. 2015).

In replicated field experiments conducted in three natural populations, Hughes et al. (2013) found that males with rare color patterns had higher reproductive success than males with common patterns. This field study did not identify the mechanism underlying this reproductive advantage; however, several laboratory studies reported that female guppies exhibit a mating preference for male color patterns that are rare or unfamiliar (Hughes et al. 1999, Eakley and Houde 2004, Zajitschek and Brooks 2008, Hampton et al. 2009, Mariette et al. 2010, Graber et al. 2015). These data suggest that female preference exerts negative frequency-dependent sexual selection. However, these behavioral experiments were conducted using laboratory-reared populations, and almost all were conducted on fish derived from a single natural population in Trinidad, or from a single feral population in Australia. Consequently, data on the prevalence of female preference for rare or unfamiliar color patterns in natural populations is lacking, as is data on among-population variation in these preferences.

Using the same experimental populations as in Hughes et al. (2013), Olendorf et al. (2006) reported that males with rare color patterns had higher survival than males with common patterns (negative frequency-dependent natural selection). A laboratory experiment subsequently reported that the killifish (*Rivulus hartii*), a natural predator of adult male guppies, is more efficient at capturing males that bear familiar color patterns (Fraser et al. 2013). This preference would produce an advantage to males with rare color patterns, which the killifish would encounter less often. These field and laboratory studies suggest that a survival advantage to rare color patterns is mediated by predator-prey interactions.

That two kinds of negative frequency-dependence have been observed in this species leaves open the possibility that both independently contribute to the maintenance of polymorphism, or that these two forms of NFDS interact. For example, a survival advantage to rare morphs could result because males with common color patterns use more energy to find or attract females, or differ in behaviors that make them more conspicuous to predators. Alternately, survival differences could impose direct or indirect selection on female preference (Kokko et al. 2007). The possibility that sexual and natural selection interact suggests a prediction that can be tested by comparing female preference for rare or unfamiliar color patterns across many natural populations:

that there should be differences in female preference for rare male color patterns associated with variation in the intensity of predation or overall mortality rate.

In Trinidad, many river drainages are characterized by ecological differences between downstream and upstream locales. Downstream habitats generally contain one or more large piscivorous fish species, and, in many drainages, these sites have more open forest canopy and high primary productivity. In contrast, upstream habitats have one primary, smaller predator of guppies, *R. hartii*, and, in many drainages, upstream sites have relatively closed canopy and low primary productivity (Grether et al. 2001, Reznick et al. 2001). *R. hartii* does occurs in both low- and high-predation sites throughout Trinidad but at higher densities in the former (Gilliam et al. 1993; Reznick et al. 1996). Because many studies have focused on the effects of predation regime on these two types of sites, they are often referred to as "low-predation" and "high-predation" sites (e.g., Endler 1995, Houde 1997, Magurran 2005). Color patterns of males from low-predation sites tend to be more conspicuous by having more large pigment-based spots compared to those of males from high-predation sites (e.g. Endler 1978; Endler 1980), but there is always considerable variation among males within any one population. These differences in predator community and the intensity of predation among populations provides an opportunity to determine if female preference for rare or unfamiliar color patterns is associated with the ecological contrast between upstream low-predation sites and downstream high-predation sites.

The experiment described here therefore had three goals. First, we sought to measure female preference for rare or unfamiliar color patterns in natural populations to determine if the preference occurs in wild fish, or if instead, it is an artifact that occurs in fish reared and tested in the laboratory. Second, we asked if the preference varies among populations. Previous studies have reported that directional female preferences for aspects of male coloration vary among populations (Houde 1987, Endler and Houde 1995), and such differences might extend to negative frequency-dependent preference. Finally, we asked if there is an association between predation regime and female preference, to test the hypothesis that natural and sexual selection favoring unusual phenotypes are not independent, but instead, one form of NFDS might be caused by the other. To address these questions, we compared female preference for rare, unfamiliar, and common color patterns across 11 natural populations in Trinidad. Similar to previous laboratory studies, color pattern rarity was defined by its frequency while familiarity depended upon whether females were likely to have prior exposure to a color pattern. Our experimental design allowed us to test both rarity and unfamiliarity simultaneously to disentangle their effects. In addition, ten of our focal populations consisted of paired high- and low-predation populations from five different river drainages, which allowed us to determine if preference was associated with differing predation regimes.

## Methods

### Populations

Experiments were performed in May of 2016 and 2017 on adult guppies from six river drainages in the Northern Range in Trinidad: the Aripo, El Cedro, Guanapo, Marianne, Paria, and Turure. For all but the Paria Tributary, we collected fish from paired high- and low-predation habitats. We note that for the Aripo, El Cedro, and Turure pairs, differences in canopy are reduced compared to the other high/low predation pairs. For each of the 11 populations (see Supplemental Table S1 for locations), we sampled fish from two to four pools that were separated from each other by at least two small waterfalls or rapids so that the fish from each pool would be unlikely to be familiar with fish from the other one to three pools (Reznick et al. 1996; Houde 1997; Olendorf et al. 2006). The number of pools sampled per population depended upon the availability of appropriate sites; we collected fish from a total of 31 pools (Supplemental Table S2). From each pool, we collected 15 to 30 males and 15 to 20 females, which accounted for 30-80% of the total population for most of the pools; refer to Table S2 where numbers of pools and females per pool are provided. Fish were caught using butterfly nets and transported in sealed Nalgene bottles containing water with Stress Coat (API) to the nearby William Beebe Tropical Research Station, located in the lower Arima valley in the Northern Range, Trinidad. Fish were separated by population and pool and allowed to acclimate in single-sex 20-40L aquaria for 24-48 hours prior to conducting female preference assays. Previous work on natural guppy populations has shown this period of time to be adequate for acclimation (Archard et al. 2008, 2009). Water exchanges of 30% were performed daily using conditioned rainwater collected on site. Fish were fed

Tetramin Tropical flake food twice daily. Behavior trials began no sooner than 30 minutes after fish were fed.

## Experimental males

This experiment was designed to examine the effects of both rarity and unfamiliarity of male color pattern on female preference, since both have been associated with female preference in previous studies. For each pool in each population, a group of four males was constructed to assess preferences of females from that pool. Three of the males were chosen from the focal pool, and one was from a pool upstream of the focal pool (at least two waterfalls or 10 meters upstream). Two of the males from the focal pool were chosen to have very similar color patterns. Similarity of color pattern was judged using previously established criteria including pattern element, color, and position (Eakley and Houde 2004, Hampton et al. 2009, Graber et al. 2015). The two males chosen to have similar patterns were designated as bearing the "common" color pattern for the group. The third male from the focal pool was chosen to have a color pattern that was unique within the group of four experimental males. This male was designated as having the “rare” color pattern for the group. Finally, the male from the upstream pool was chosen to have a color pattern different from the common and rare patterns. This male’s color pattern was therefore both rare within the group of four males, and he was likely to be individually unfamiliar to the focal female. (Note that guppies readily learn to recognize individual conspecifics (Griffiths and Magurran 1997, Kelley et al. 1999, Valero et al. 2009, Mariette et al. 2010)). Hereafter we will refer to this male as “unfamiliar” for brevity. This method of constructing male groups does not guarantee that color patterns designated as "rare" or "common" in a group were also rare or common in the natural population from which the fish were sampled. However, previous studies have shown that preference for rare and unfamiliar color patterns operates similarly over long and short timescales; that is, females prefer males bearing rare or unfamiliar color patterns when the treatment exposures occur over time periods ranging from a few minutes to several days (Hughes et al. 1999, Zajitschek et al. 2006, Zajitschek and Brooks 2008, Hampton et al. 2009, Jordan and Brooks 2010, Mariette et al. 2010, Graber et al. 2015, Daniel et al. 2019). Based on these prior studies, the exposure period used in this experiment was sufficient to stimulate female discrimination among common, rare, and unfamiliar color patterns. The four males within a group were chosen to be approximately the same size (maximum difference between males within a group = 2.78 mm, mean=0.13±0.06 mm); however, male size was also included as a covariate in the statistical analysis (see below).

Quantitative analyses of color patterns were performed on the photographs described below (see Male phenotypes) to confirm classification of color pattern types using the *patternize* (Van Belleghem et al. 2018) and *ade4* (Chessel et al. 2004) packages in R V3.5.3. The *patRegK* function in the *patternize* package was used to align homologous structures, allowing for superimposition of the images of the four males from each pool; that is, alignment was performed separately for each group of males that were used to assess female preference. This function used *k*-means clustering of RGB values as an unsupervised method to identify colors and to generate matrices containing binary data (presence/absence) for each color at each pixel coordinate. Clusters were specified by the user beginning with k=7 and increased until black and orange color clusters were identified; we selected black and orange because a previous study found a female response to these colors in three of the populations used in this experiment (Endler and Houde 1995). We then computed distance matrices for both colors with the *dist.binary* function in the *ade4* package (Chessel et al. 2004). This function calculated a dissimilarity coefficient using the binary data matrices for each color cluster identified by the *patRegK* function. We used two different methods (Method 1 = Jaccard Index, Method 8 = Sokal & Sneath) to evaluate color pattern dissimilarity (Gower and Legendre 1986). These dissimilarity metrics indicated that the color patterns of the males classified as common within the same experimental group were most similar to each other (had the lowest dissimilarity metric), and they indicated that the unfamiliar and rare patterns were less similar to the common pattern and to each other than the males with the common pattern were to each other (Supplemental Table S3). We also performed a Principal Components Analysis (PCA) on the orange and black color clusters using *patPCA:patternize* and computed a Euclidean distance matrix on the resulting PCA scores for orange and black using the *dist* function (R Core Team 2019)  (Supplemental Figure S1). Overall, the two males within each group classified as common had color patterns that were closest in PCA space for both orange and black (Supplemental Table S3) while rare and unfamiliar males were nearly equally distant from the common color patterns. We concluded that our classification of color patterns successfully captured similarity and dissimilarity among the four males within each group, and that males classified as rare were at least as dissimilar to the common pattern as were the males with unfamiliar patterns that originated from adjacent pools.

### Behavioral trials

Before behavior assays commenced, clean conditioned rainwater was added to the 42.5L experimental tank to a depth of 14cm. This water was replaced for each new assayed pool. A single standard household fluorescent light bar (40 W) was centered over the experimental tank and suspended 30 cm above the surface of the water. Additional illumination was provided by an incandescent light bulb (40 W) suspended 1 meter above the surface of the water. Some natural light also entered the room over the top of a dividing wall (2.5m above the tank) and through a shuttered door. The experimental tank was covered on three sides with gray cardboard to provide a neutral background and minimize visual disturbance during the experiment. Observations were scored live, by an observer seated at eye level, 1m from the open side of the experimental tank.

The experimental tank was divided in to five separate compartments (one central and two on each side; Figure 2) by clear perforated plastic dividers (Supplemental Figure S2) that allowed transmission of visual and chemical cues. Each side compartment (for the four males) measured 15.5cm x 16cm. The remaining central area (17cm x 31cm) was for the focal female and companion females. Within the central compartment, the regions within 6 cm (approximately three female body lengths) of the partition separating males from females were marked to identify when the focal female was in close proximity to males. This criterion for close proximity was similar to that of previous studies (Kodric-Brown 1985, Houde 1988, Lafleur et al. 1997, Kodric-Brown and Nicoletto 2001, Zajitschek and Brooks 2010). This region was designated as ‘in zone’ of the adjacent compartment containing a male. The remaining area of the central female compartment was designated as a ‘neutral zone’. The observer (JJV) was blind to the compartment location of each male type. Additionally, only the color patterns of males located in the compartments closest to the observer were clearly visible during the time of the trial making it impossible to determine which two of the four color patterns were most similar (and therefore common).

A focal female and four sexually mature companion females were selected from the same focal pool as the males used in a behavior trial. Companion females were selected to be sexually mature but smaller in size to allow for identification of the focal female. We used companion females because isolated female guppies often behave abnormally (pers. obs.). Because of the small census size of some pools (especially in low-predation sites) and to maximize the number of focal females that could be sampled from each pool, the same companion females and the same four experimental males were used for all the focal females tested from a pool. Males and companion females were placed in their respective compartments of the experimental tank for a five-minute acclimation period prior to the addition of the first focal female for each group of males.

Observation periods were 15 minutes and began no earlier than 8:45am and ended by 7:00pm (time of day included in the statistical model, see below). If the focal female remained motionless or did not leave the neutral zone for two minutes following her addition to the experimental tank, she was removed and replaced with a new focal female. At the completion of the observation period, the focal female was removed from the experimental tank. The experimental males were removed from the experimental tank, then placed in a randomly selected compartment, and given a five-minute acclimation period prior to the addition of a new focal female from their pool. We tested a mean of 6.73±1.40 females per pool (range 3-10 females, see Supplementary Table S2).

We recorded the following female behaviors using JWatcher (Version 1.0) event recording software (Blumstein et al. 2006): motionless, swimming, glass running, in zone, in neutral zone, and orienting toward males (see Table 1 for behavior definitions). Both association time (time in zone) and orienting have been used to measure female preference in this species (Bischoff et al. 1985, Houde and Torio 1992, Kodric-Brown 1992, Brooks and Caithness 1995, Houde 1997, Rosenqvist and Houde 1997, Houde and Hankes 1997, Hibler and Houde 2006, Hampton et al. 2009), and these behaviors predict male mating success (Bischoff et al. 1985, Houde 1988, Kodric-Brown 1992). As our measure of female interest in males, we used the total time females spent oriented towards a male; we refer to this measure as attention. We did not use time in zone in a male's compartment as a measure of female interest because preliminary analysis indicated that females spent more time in zone in some compartments than in others

($F_{3,193} = 8.28$, $p<0.0001$), perhaps because of sources of natural light that could not be controlled. To account for this compartment effect, we used time in zone in each compartment as a covariate in the statistical model (see below). The duration that females spent glass running or out of zone were not included in the statistical analysis of female attention.

We assessed attention toward each type of male color pattern for females sampled from 31 different pools distributed across six rivers and two predation regimes (Supplemental Table S2). Observations from 30 pools were included in the final analysis; one pool (Pool C) of the Marianne high-predation population was not included (N=5 females). The four experimental males from this pool did not survive long enough following the behavior assay to allow us to phenotype them (see below). All males from all other pools survived and appeared healthy when photographed up to 24 hours after their final behavior trial. Observations of one female from the Guanapo high-predation population (Pool A) were not used because the female only entered into zone of three of the four male compartments and therefore may have failed to assess one of the males. Our final analysis therefore included a total sample size of N=192 females.

## Male phenotypes

Male size and color data were collected from digital images. Experimental males were lightly anesthetized with buffered MS222 and photographed (Canon EOS 5D, 100mm macro lens) <24 hours after the behavior assay. A prepared stage that included size and color standards was placed within a Photo Cube lighting tent and illuminated by two 65-watt daylight fluorescent full spectrum light bulbs (Fovitec StudioPRO) positioned on opposite sides of the stage and angled down approximately 30 degrees from the plane of the camera lens. Images were taken in RAW format and converted to TIFFs in Adobe Photoshop CC 2018. For many but not all Trinidadian populations, female guppies prefer males with a greater area of orange spots (Houde 1987, Houde and Endler 1990, Endler and Houde 1995). We therefore included the amount of orange in a male's color pattern as a covariate in our analysis to distinguish this directional color preference from preference for rare or unfamiliar patterns. To do this, we measured the total area of orange using the *Threshold Color* plugin in ImageJ 1.8 software. Hue, Saturation, and Brightness (HSB) color space was used to select orange pixels and determine the total area of orange for each male. The HSB threshold values used to select pixels were set to the following ranges: hue 11-35, saturation 80-255, brightness 50-250. These values successfully selected the orange color standard and omitted the red and yellow standards; in addition, these values selected all appropriate orange areas on several test fish. Male standard length (SL) was measured from the tip of the snout to the posterior end of the caudal peduncle. Total area of orange and standard length were included in the statistical analysis (see below).

## Statistical analysis

We assessed whether females differed in their attention toward unfamiliar, rare, or common male color patterns, and whether any such effects varied by river or by predation regime. To do so, we used generalized linear mixed models with repeated measures in *Proc Mixed* of SAS version 9.4 (SAS Institute Inc., Cary, NC). In addition to male type, river, and predation regime, the model included measures of male orange color, standard length, year (2016 or 2017), and covariates to account for variation in time in zone (described above) and time of day. Time of day was never significant ($P>0.22$) and was therefore removed from the final model. Initial models also included all interactions up to three-way interactions between categorical effects (male type, river, predation regime, and year) except for interactions including both river and predation regime (one river, the Paria Tributary, did not have a high-predation contrast) and interactions including year and predation regime (only the Paria Tributary was tested in both years). Each continuous covariate and its two- and three-way interactions with the categorical variables were also included in initial models. Non-significant interaction terms between fixed effects were eliminated from models when $P>0.20$, using backward elimination to arrive at the final model. We report results only of the final statistical model.

We also determined if female attention varied among populations, irrespective of predation regime or drainage. To do so, we used a statistical model identical to the one described above, except that a unique population ID was used as a fixed effect in place of river and predation regime. Model simplification proceeded as described above.

For all statistical tests, we transformed the dependent variable (female attention) to conform to assumptions of the analysis (a fourth-root transformation produced residuals that were normally distributed and homoscedastic). Some covariates were also transformed to conform to model assumptions: area of male orange color (square root transformed), and time in zone (cube-root transformed). Each continuous fixed effect was also standardized (mean = 0, standard deviation = 1) to improve interpretability of covariate effects and interactions (Schielzeth 2010).

To account for the hierarchical structure of the data (replicate pools were sampled within predation regimes that were nested with river drainages), we used the *random* statement in Proc Mixed to specify a random effect for pool nested within river and predation regime. To account for the fact that the same males (and their type assignments) were used with multiple females from the same pool, we used a random effect of male type by pool interaction; this term provides the appropriate error term for testing the fixed effect of male type. To account for repeated measures on male groups and on females (female behavior was measured with each of the four males in her trial), we used female ID and compartment as the subject terms in a *repeated* statement. We used the *type=un@cs* option to specify an unstructured covariance matrix for repeated measures on females and a compound symmetric covariance structure for repeated measures on male groups; this structure provided the best fit using the AICC criterion. The option *group=year* was also used in the repeated statement to allow for separate covariance estimates for the two experimental periods (years). We used restricted maximum likelihood estimation and the Kenward-Roger method for estimating denominator degrees of freedom. The Kenward-Roger method accounts for random effects, adjusts for small-sample bias in parameter estimates and standard errors, and is appropriate for correlated error models (Littell et al. 2006, Bell et al. 2013, 2014).

To assess differences between levels of fixed effects that comprised more than two levels, post hoc comparisons between least-square means were adjusted using the *simulate(cvadjust)* option in *Proc Mixed* to control for multiple tests. Figures illustrating group differences are presented on the untransformed scale for ease of interpretation.

## Results

We observed a significant overall effect of male color pattern type on the female attention toward males (Table 2, Figure 3; see Table S4 for model parameter estimates). After correcting for orange area and the other covariates, post-hoc pairwise comparisons show that females spent 18.6% more time attending to males bearing unfamiliar compared to those bearing common patterns (mean ± SE of 32.34±2.53 and 27.26±2.37 seconds respectively, $t_{42.6} = -3.23$, adjusted P=0.006, Table S5). Females also spent 10.9% more time attending to males having rare compared to common color patterns, but this difference was not significant (mean ± SE of 30.24±2.60 seconds for rare patterns, $t_{44.8}=-1.54$, P=0.13, adjusted P=0.28; Table S5). Females attended more to males having unfamiliar versus rare color patterns (6.9%), however this difference was not significant ($t_{65}=-1.42$, P=0.16, Adjusted P=0.34; Table S5).

The increased attention females paid to unfamiliar compared to common male color patterns was not influenced by river, predation regime, male size or male color. That is, female attention to different types of color patterns did not interact significantly with any other predictors in the model. However, overall female attention was influenced by a few other predictors. Females attended more to males with more orange area in their color pattern (Table 2, Figure 4). On average, the top 5% of males with the most orange color received 69.5%~~50.5%~~ more attention than ~~those with~~ the 5% of males with the least amount of orange. Predation regime also significantly affected female attention (Table 2). Females from high-predation populations attended more to males than did females from low-predation populations (mean ± SE of 34.28±3.15 and 25.61±2.61 seconds respectively, Figure 5).

~~Male SL was significantly associated with female attention, with l~~Females ~~also~~ paid more attention to larger males, with the largest 5% of males receiving 72.0%XX more attention ~~on average~~than the smallest 5% of males (Table 2, Figure 6).~~,~~ Year (2016 vs 2017) was also a significant predictor of female attention (Table 2), with~~. F~~females ~~attended significantly~~ paying more attention to males in 2016 than 2017 (mean ± SE of 41.35±4.31 and 18.54±4.80 seconds respectively, Figure S3). This difference might have arisen because different populations were sampled in different years.

**Commented [KAH1]:** Not sure how you calculated this for orange. Just eyeballing the Figure, I calculated 79%, but that seems kind of high. Also, it seems as if the slope should be higher for the orange figure than for the SL figure (based on the estimates in the Table), but visually they look identical.

**Commented [JV2R1]:** I did the calculations and added the text. However, the sentence on line 423 to 428 was odd, so I

We also asked if female attention for unfamiliar, rare, and common male patterns varied among populations, irrespective of predation regime or drainage. For a model where a unique population ID was used in place of river and predation regime designations, female preference for unfamiliar, rare, and common patterns was not influenced by the identity of the population (Table S6, see Table S7 for parameter estimates). In this model, overall female attention was significantly affected by an interaction between year and male SL (Table S6). This interaction is due to SL having no relationship to female attention in year 1 (slope = -0.01±- 0.04, Table S7), but having a positive association in year 2 (slope=0.15±- 0.07, Table S7). As for overall attention, this difference presumably arose because different populations were sampled in different years.

## Discussion

Previous field experiments demonstrated that males bearing rare color patterns had higher reproductive success than those bearing common color patterns (Hughes et al. 2013). Evidence from several laboratory studies suggests that this negative frequency-dependent reproductive success is mediated by female preference for males bearing rare or unfamiliar color patterns (Farr 1977, Hughes et al. 1999, Eakley and Houde 2004, Zajitschek et al. 2006, Zajitschek and Brooks 2008, Hampton et al. 2009, Mariette et al. 2010, Graber et al. 2015). Our experiment with wild-caught guppies supports this hypothesis: females paid more attention to males with color patterns that were unfamiliar and less attention to males with common color patterns. Note that color patterns that were unfamiliar were also rare in this experiment. This pattern is similar to that reported by Zajitschek and Brooks (2008), who compared female preference for rare, unfamiliar, and common color patterns using laboratory-reared guppies from a feral population in Australia. In that study, rarity, but not familiarity, predicted female preference. However, males with rare color patterns were also unfamiliar in that experiment, so the preferred male type, as in our study, was both rare and unfamiliar. Taken together, the results of these studies suggest that both rarity of color patterns and unfamiliarity contribute to male attractiveness.

Other patterns we observed for female preference are similar to those reported for laboratory-reared guppies. We found that high-predation females paid more attention to males compared to low-predation females. Previous laboratory studies reported differences in the strength of female preferences among populations, and suggested that preferences might vary with predation regime (Endler and Houde 1995, Godin and Briggs 1996). For example, Godin and Briggs (1996) reported that females from one high-predation site (Quare) spent more time associated with males than did females from a low-predation (Paria) site. One explanation for this pattern could be differences in male courtship rate which then influence female behavior. Several studies reported that females prefer males with higher display rates (Farr 1980, Nicoletto 1993, Kodric-Brown and Nicoletto 1996, 2001) and that high-predation males court at higher rates (Farr 1975, Magurran and Seghers 1994). In our study, we were unable to score male courtship behavior due to the difficulty of scoring displays of males located in the two compartments furthest from the observer. Future studies should include measures of male courtship rates to disentangle the roles of these potential causes of greater attention by high-predation females.

Wild guppies in our experiment exhibited a directional preference for more orange color, similar to previous studies of laboratory-reared guppies (e.g., Houde and Endler 1990; Kodric-Brown and Nicoletto 1996; Rosenqvist and Houde 1997; Pitcher et al. 2003). As in previous work, this preference was independent of that for rarity and unfamiliarity (Zajitschek et al. 2006, Zajitschek and Brooks 2008, Hughes et al. 2013, Graber et al. 2015). These results suggest that both the frequency-dependent female preference and preference for specific colors in wild guppies are robustly reflected in studies of laboratory-reared populations.

Male guppy color patterns are known to vary within and among river drainages in association with important ecological factors, including predation (Endler 1983, Grether et al. 1999, 2001). Our sampling of paired high- and low-predation populations allowed us to determine if female preference for rare or unfamiliar color patterns varied across river drainages or between high- and low-predation populations. Although females from high-predation sites paid more attention to males overall, this effect did not interact with male type. We cannot rule out that such an effect would emerge in a larger study. Nevertheless, taken at face value, this result does not support the hypothesis that reproductive and survival differences are linked (which could happen, e.g., because less preferred males experience greater mortality risk or because survival differences impose indirect selection on female preference). Rather, consistency of this preference across ecologically diverse populations suggests that the evolutionary processes leading to it are relatively uniform.  For example, a recent study reports that female preference for unfamiliar male color patterns meets the formal criteria for habituation to sensory stimuli, a simple form of learning that is ubiquitous among animals (Daniel et al. 2019). Habituation is thought to be highly conserved because it allows organisms to filter repetitive sensory input and focus on novel stimuli likely to be more biologically relevant (Groves and Thompson 1970, Rankin et al. 2009).

Our results suggest that, whatever its evolutionary origin, female preference for rare and unfamiliar male color patterns exerts NFDS on male coloration in many or most populations in the Northern Range of Trinidad. The prevalence and consistency of this preference suggests that it is the mechanism underlying the rare-male reproductive advantage observed in natural populations of guppies (Hughes et al. 2013). NFDS is a powerful force for maintaining genetic variation (Ayala and Campbell 1974), and models of negative frequency-dependent female preference indicate that it can readily maintain male-limited polymorphism (Kokko et al. 2007). These models show that preference for rarity maintains male polymorphism even when the preference is costly and when females are prevented from fully expressing preference, as might occur when male behavior or predator threat modulates the outcome of mating interactions. The ubiquity of female preference for rare and unfamiliar male color patterns in wild guppy populations therefore suggests that this preference is a major driver of high genetic diversity in this ecologically important trait.

## Conclusions

How genetic variation in ecologically important traits is maintained when natural selection and genetic drift tend to erode it is a long-standing paradox in evolutionary biology (Lewontin 1974, Charlesworth and Hughes 2000, Charlesworth 2015). Results reported here support the hypothesis that female preference for rare and unfamiliar male color patterns promotes high genetic diversity in male coloration in guppies. An important unresolved question is whether this female preference is also responsible for the survival advantage that rare (Olendorf et al. 2006) and unfamiliar (Fraser et al. 2013) male guppies experience, or whether two independent forms of NFDS operate in this system.

## Ethics statement

The research presented was described in Animal Research Protocol No. 1442 approved on 29 October 2014 and Protocol No. 1740 approved on 16 October 2017, by the Animal Care and Use Committee of Florida State University.

## Literature Cited

Archard, G. A., I. C. Cuthill, and J. C. Partridge. 2009. Light environment and mating behavior in Trinidadian guppies (*Poecilia reticulata*). Behavioral Ecology and Sociobiology 64:169–182.

Archard, G. A., I. C. Cuthill, J. C. Partridge, and C. Van Oosterhout. 2008. Female guppies (*Poecilia reticulata*) show no preference for conspecific chemosensory cues in the field or an artificial flow chamber. Behaviour 145:1329–1346.

Ayala, F. J., and C. A. Campbell. 1974. Frequency-dependent selection. Annual Review of Ecology and Systematics 5:115–138.

Barton, N. H., and M. Turelli. 1989. Evolutionary quantitative genetics: how little do we know? Annual review of genetics 23:337–370.

Bell, B. A., M. Ene, W. Smiley, and J. A. Schoeneberger. 2013. A multilevel model primer using SAS PROC MIXED. SAS Global Forum.

Bell, B. A., W. Smiley, M. Ene, P. R. Sherlock, and G. L. Blue. 2014. An intermediate primer to estimating linear multilevel models using SAS PROC MIXED. SAS Proceedings:1–13.

Van Belleghem, S. M., R. Papa, H. Ortiz-Zuazaga, F. Hendrickx, C. D. Jiggins, W. Owen McMillan, and B. A. Counterman. 2018. patternize: An R package for quantifying colour pattern variation. Methods in Ecology and Evolution 9:390–398.

Bischoff, R. J., J. L. Gould, and D. I. Rubenstein. 1985. Tail size and female choice in the guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology 17:253–255.

Bleay, C., T. Comendant, and B. Sinervo. 2007. An experimental test of frequency-dependent selection on male mating strategy in the field. Proceedings. Biological sciences / The Royal Society 274:2019–2025.

Blumstein, D. T., C. S. Evans, and J. C. Daniels. 2006. JWatcher (V1.0). http://www.jwatcher.ucla.edu.

Brooks, R. C., and J. A. Endler. 2001. Direct and indirect sexual selection and quantitative genetics of male traits in guppies (*Poecilia reticulata*). Evolution 55:1002–1015.

Brooks, R., and N. Caithness. 1995. Manipulating a seemingly non-preferred male ornament reveals a role in female choice. Proceedings of the Royal Society B: Biological Sciences 261:7–10.

Cain, A. J., and P. M. Sheppard. 1950. Selection in the polymorphic land snail *Cepaea nemoralis*. Heredity 4:275–294.

Charlesworth, B. 2015. Causes of natural variation in fitness: evidence from studies of *Drosophila* populations. Proceedings of the National Academy of Sciences 112:E1049–E1049.

Charlesworth, B., and K. A. Hughes. 2000. The Maintenance of Genetic Variation in Life-History Traits. Pages 369–392 *in* R. S. Singh and C. B. Krimbas, editors. Evolutionary Genetics: From Molecules to Morphology. Cambridge University Press, Cambridge.

Chessel, D., A.-B. Dufour, and J. Thioulouse. 2004. The ade4 package-I-One-table methods. R news 4:5–10.

Daniel, M. J., L. Koffinas, and K. A. Hughes. 2019. Habituation underpins preference for mates with novel phenotypes in the guppy. Proceedings of the Royal Society B: Biological Sciences 286:20190435.

Delph, L. F., and J. K. Kelly. 2014. On the importance of balancing selection in plants. New Phytologist 201:45–56.

Eakley, A. L., and A. E. Houde. 2004. Possible role of female discrimination against “redundant” males in the evolution of colour pattern polymorphism in guppies. Proceedings. Biological sciences / The Royal Society 271 Suppl:S299–S301.

Endler, J. A. 1978. A Predator’s View of Animal Color Patterns. Pages 319–364 *in* M. K. Hecht, W. C. Steere, and B. Wallance, editors. Evolutionary Biology. Springer, Boston.

Endler, J. A. 1980. Natural selection on color patterns in *Poecilia reticulata*. Evolution 34:76–91.

Endler, J. A. 1983. Natural and sexual selection on color patterns in poeciliid fishes. Environmental Biology of Fishes 9:173–190.

Endler, J. A. 1995. Multiple-trait coevolution and environmental gradients in guppies. Trends in Ecology and Evolution 10:22–29.

Endler, J. A., and A. E. Houde. 1995. Geographic variation in female preferences for male traits in *Poecilia reticulata*. Evolution 49:456–468.

Farr, J. 1977. Male rarity or novelty, female choice behavior, and sexual selection in the guppy, *Poecilia reticulata* Peters (Pisces: Poeciliidae). Evolution 31:162–168.

Farr, J. A. 1975. The role of predation in the evolution of social behavior of natural populations of the guppy, *Poecilia reticulata* (Pisces: Poeciliidae). Evolution 29:151–158.

Farr, J. A. 1980. Social behavior patterns as determinants of reproductive success in the guppy, *Poecilia reticulata* Peters (Pisces: Poeciliidae) an experimental study of the effects of intermale competition, female choice, and sexual selection. Behaviour 74:38–90.

Fraser, B. A., K. A. Hughes, D. N. Tosh, and F. H. Rodd. 2013. The role of learning by a predator, *Rivulus hartii*, in the rare-morph survival advantage in guppies. Journal of Evolutionary Biology 26:2597–2605.

Gigord, L. D., M. R. Macnair, and A. Smithson. 2001. Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo. Proceedings of the National Academy of Sciences of the United States of America 98:6253–6255.

Gilliam, J. F., D. F. Fraser, and M. Alkins-Koo. 1993. Structure of a Tropical Stream Fish Community: A Role for Biotic Interactions. Ecology 74:1856–1870.

Godin, J. G. J., and S. E. Briggs. 1996. Female mate choice under predation risk in the guppy. Animal Behaviour 51:117–130.

Gower, J. C., and P. Legendre. 1986. Metric and Euclidean properties of dissimilarity coefficients. Journal of Classification 3:5–48.

Graber, R. E., M. Senagolage, E. Ross, A. E. Houde, and K. A. Hughes. 2015. Mate preference for novel phenotypes: a fresh face matters. Ethology 121:17–25.

Grether, G. F., J. Hudon, and D. F. Millie. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. Proceedings of the Royal Society B: Biological Sciences 266:1317.

Grether, G. F., D. F. Millie, M. J. Bryant, D. N. Reznick, and W. Mayea. 2001. Rain forest canopy cover, resource availability, and life history evolution in guppies. Ecology 82:1546–1559.

Griffiths, S. W., and A. E. Magurran. 1997. Familiarity in schooling fish: How long does it take to acquire? Animal Behaviour 53:945–949.

Groves, P. M., and R. F. Thompson. 1970. Habituation: A dual-process theory. Psychological Review 77:419–450.

Hampton, K. J., K. A. Hughes, and A. E. Houde. 2009. The allure of the distinctive: reduced sexual responsiveness of female guppies to ‘redundant’ male colour patterns. Ethology 115:475–481.

Hibler, T. L., and A. E. Houde. 2006. The effect of visual obstructions on the sexual behaviour of guppies: the importance of privacy. Animal Behaviour 72:959–964.

Houde, A. E. 1987. Mate choice based upon naturally occurring color-pattern variation in a guppy population. Evolution 41:1–10.

Houde, A. E. 1988. The effects of female choice and male-male competition on the mating success of male guppies. Animal Behaviour 36:888–896.

Houde, A. E. 1997. Sex, Color, and Mate Choice in Guppies. Princeton University Press, Princeton, NJ.

Houde, A. E., and J. A. Endler. 1990. Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. Science 248:1405–1408.

Houde, A. E., and M. A. Hankes. 1997. Evolutionary mismatch of mating preferences and male colour patterns in guppies. Animal Behaviour 53:343–351.

Houde, A. E., and A. J. Torio. 1992. Effect of parasitic infection on male color pattern and female choice in guppies. Behavioral Ecology 3:346–351.

Hughes, K. A., L. Du, F. H. Rodd, and D. N. Reznick. 1999. Familiarity leads to female mate preference for novel males in the guppy, *Poecilia reticulata*. Animal behaviour 58:907–916.

Hughes, K. A., A. E. Houde, A. C. Price, and F. H. Rodd. 2013. Mating advantage for rare males in wild guppy populations. Nature 503:108–10.

Hughes, K. A., F. H. Rodd, and D. N. Reznick. 2005. Genetic and environmental effects on secondary sex traits in guppies (*Poecilia reticulata*). Journal of Evolutionary Biology 18:35–45.

Hugie, D. M., and D. B. Lank. 1997. The resident’s dilemma: a female choice model for the evolution of alternative mating strategies in lekking male ruffs (*Philomachus pugnax*). Behavioral Ecology 8:218–225.

Indermaur, A., A. Theis, B. Egger, and W. Salzburger. 2018. Mouth dimorphism in scale-eating cichlid fish from Lake Tanganyika advances individual fitness. Evolution 72:1962–1969.

Jordan, L. A., and R. C. Brooks. 2010. The lifetime costs of increased male reproductive effort: courtship, copulation and the Coolidge effect. Journal of Evolutionary Biology 23:2403–2409.

Kelley, J. L., J. A. Graves, and A. E. Magurran. 1999. Familiarity breeds contempt in guppies. Nature 401:661–662.

Kodric-Brown, A. 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology 17:199–205.

Kodric-Brown, A. 1992. Male dominance can enhance mating success in guppies. Animal Behaviour 44:165–167.

Kodric-Brown, A., and P. F. Nicoletto. 1996. Consensus among females in their choice of males in the guppy *Poecilia reticulata*. Behavioral Ecology and Sociobiology

39:395–400.

Kodric-Brown, A., and P. F. Nicoletto. 2001. Female choice in the guppy (*Poecilia reticulata*): the interaction between male color and display. Behavioral Ecology and Sociobiology 50:346–351.

Kokko, H., M. D. Jennions, and A. E. Houde. 2007. Evolution of frequency-dependent mate choice: keeping up with fashion trends. Proceedings. Biological sciences / The Royal Society 274:1317–1324.

Lafleur, D. L., G. A. Lozano, and M. Sclafani. 1997. Female mate-choice copying in guppies, *Poecilia reticulata*: A re-evaluation. Animal Behaviour 54:579–586.

Leffler, E. M., K. Bullaughey, D. R. Matute, W. K. Meyer, L. Ségurel, A. Venkat, P. Andolfatto, and M. Przeworski. 2012. Revisiting an old riddle: What determines genetic diversity levels within species? PLoS Biology 10.

Lewontin, R. C. 1974. The Genetic Basis of Evolutionary Change. Columbia University Press, New York.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger, and O. Schabenberger. 2006. SAS for Mixed Models. Second. SAS Institute Inc., Cary, NC.

Magurran, A. E. 2005. Evolutionary Ecology: The Trinidadian Guppy. Oxford University Press, New York.

Magurran, A. E., and B. H. Seghers. 1994. Sexual conflict as a consequence of ecology:

evidence from guppy, *Poecilia reticulata* , populations in Trinidad. Proceedings of the Royal Society of London. Series B: Biological Sciences 255:31–36.

Mariette, M. M., S. R. K. Zajitschek, C. M. Garcia, and R. C. Brooks. 2010. The effects of familiarity and group size on mating preferences in the guppy, *Poecilia reticulata*. Journal of Evolutionary Biology 23:1772–1782.

Mitchell-Olds, T., J. H. Willis, and D. B. Goldstein. 2007. Which evolutionary processes influence natural genetic variation for phenotypic traits? Nature reviews. Genetics 8:845–856.

Mokkonen, M., H. Kokko, E. Koskela, J. Lehtonen, T. Mappes, H. Martiskainen, and S. C. Mills. 2011. Negative frequency-dependent selection of sexually antagonistic alleles in *Myodes glareolus*. Science 334:972–974.

Nicoletto, P. F. 1993. Female sexual response to condition-dependent ornaments in the guppy, *Poecilia reticulata*. Animal Behaviour 46:441–450.

Olendorf, R., F. H. Rodd, D. Punzalan, A. E. Houde, C. Hurt, D. N. Reznick, and K. A. Hughes. 2006. Frequency-dependent survival in natural guppy populations. Nature 441:633–6.

Pitcher, T. E., B. D. Neff, F. H. Rodd, and L. Rowe. 2003. Multiple mating and sequential mate choice in guppies: females trade up. Proceedings. Biological sciences / The Royal Society 270:1623–1629.

R Core Team. 2019. R: A Language and Environment for Statistical Computing. Vienna, Austria.

Rankin, C. H., T. Abrams, R. J. Barry, S. Bhatnagar, D. F. Clayton, J. Colombo, G. Coppola, M. A. Geyer, D. L. Glanzman, S. Marsland, F. K. McSweeney, D. A. Wilson, C. F. Wu, and R. F. Thompson. 2009. Habituation revisited: An updated and revised description of the behavioral characteristics of habituation. Neurobiology of Learning and Memory 92:135–138.

Reznick, D., M. J. Butler IV, and H. Rodd. 2001. Life-history evolution in guppies. VII. The comparative ecology of high- and low-predation environments. The American Naturalist 157:126–140.

Reznick, D. N., M. J. Butler, F. H. Rodd, and P. Ross. 1996a. Life-history evolution in guppies (*Poecilia reticulata*) VI. Differential mortality as a mechanism for natural selection. Evolution 50:1651–1660.

Reznick, D. N., F. H. Rodd, and M. Cardenas. 1996b. Life-History evolution in guppies (*Poecilia reticulata* : Poeciliidae ). IV . Parallelism in life-history phenotypes. American Naturalist 147:319–338.

Rivkin, L. R., A. L. Case, and C. M. Caruso. 2015. Frequency-dependent fitness in gynodioecious *Lobelia siphilitica*. Evolution 69:1232–1243.

Rosenqvist, G., and A. E. Houde. 1997. Prior exposure to male phenotypes influences mate choice in the guppy, *Poecilia reticulata*. Behavioral Ecology 8:194–198.

Schielzeth, H. 2010. Simple means to improve the interpretability of regression coefficients. Methods in Ecology and Evolution 1:103–113.

Shuster, S. M., and M. J. Wade. 1991. Equal mating success among male reproductive strategies in a marine isopod. Nature 350:608–610.

Sinervo, B., and C. M. Lively. 1996. The rock–paper–scissors game and the evolution of alternative male strategies. Nature 380:240–243.

Takahashi, Y., J. Yoshimura, S. Morita, and M. Watanabe. 2010. Negative frequency-dependent selection in female color polymorphism of a damselfly. Evolution 64:3620–3628.

Troth, A., J. R. Puzey, R. S. Kim, J. H. Willis, and J. K. Kelly. 2018. Selective trade-offs maintain alleles underpinning complex trait variation in plants. Science 361:475–478.

Valero, A., A. E. Magurran, and C. M. Garcia. 2009. Guppy males distinguish between familiar and unfamiliar females of a distantly related species. Animal Behaviour 78:441–445.

Valvo, J., F. H. Rodd, and K. A. Hughes. 2019. Data from: Consistent female preference for rare and unfamiliar male color patterns in wild guppy populations. Dryad Digital Repository. (doi:10.5061/dryad.70v5js4).

Zajitschek, S. R. K., and R. C. Brooks. 2008. Distinguishing the effects of familiarity, relatedness, and color pattern rarity on attractiveness and measuring their effects on sexual selection in guppies (*Poecilia reticulata*). The American naturalist 172:843–

854.

Zajitschek, S. R. K., and R. C. Brooks. 2010. Inbreeding depression in male traits and preference for outbred males in *Poecilia reticulata*. Behavioral Ecology 21:884–891.

Zajitschek, S. R. K., J. P. Evans, and R. C. Brooks. 2006. Independent effects of familiarity and mating preferences for ornamental traits on mating decisions in guppies. Behavioral Ecology 17:911–916.

## Figure Legends

**Figure 1.** (A) Example of female Trinidadian guppy which lacks color polymorphism. Variation in male-limited guppy color patterns in the (B) low-predation Paria Tributary, (C) high-predation Marianne River, and (D) low-predation Guanapo River populations.

**Figure 2.** Diagram of experimental aquarium used in behavior assay. The dotted lines represent a perforated UV-permissive plastic divider to allow transfer of visual and chemical cues. A focal female and four companion females were placed in the central compartment. Experimental males were placed in each of the four end compartments, with male position randomly assigned in each focal female assay. See text for explanation of male types.

**Figure 3.** LS means ± SE on the untransformed scale showing the total time females (N=192) spent attending to males having three possible types of color patterns (unfamiliar, rare, and common).

**Figure 4.** Females directed more attention to males with a greater area of orange color in their color pattern. Each point represents the average attention time a male (N=120) received from all focal females in his assay. Transformed attention time is reported on the

y axis. Orange area is square-root transformed standardized (mean=0, standard deviation =1).

**Figure 5.** LS means ± SE on the untransformed scale. Females from high-predation populations (N=83) paid significantly more attention to males than females from low-predation populations (N=109).

**Figure 6.** Females directed more attention to males with a larger standard length. Each point represents the average attention time a male (N=120) received from all focal females in his assay. Transformed attention time is reported on the y axis. Male standard length (SL) is standardized (mean=0, standard deviation =1).

**Supplemental Figure S1.** Shown here is an example of the Principal Components Analysis (PCA) plots from the *patPCA* function in the *patternize* package. We performed a PCA for the orange (A) and black (B) clusters for one pool from the Paria population (PRLP17 Pool A). The four males bearing the three color pattern types are represented by the symbols: gold squares (common), green circles (rare), and purple triangles (unfamiliar). The PCA was performed on the binary (presence/absence) data generated from the *patRegK* function in *patternize*. This function was used to align homologous structures and allowed for the superimposition of the four male color patterns from each pool. We then used *k*-means clustering as an unsupervised approach to determine color boundaries. Clusters were specified by the user (JJV) beginning with k=7 and increased until black and orange color clusters were identified. The number of clusters varied by pool with the maximum clusters specified of k=13. The cartoon outline identifies the aligned location on each fish where the specified color cluster was found for at least one of the four male group members. The colors ranging from blue through black to red on the cartoon represent the predicted changes in color pattern along each principal component axis; positive values indicate a higher predicted presence of color at that location and negative values indicate the absence of the color.

**Supplemental Figure S2.** Image of UV permissible clear plastic divider on a brown background. This material was used to create five compartments which separated individual males from each other and from females in the experimental tank design. Holes are 2mm in diameter and 4mm apart allowing the exchange of chemical and visual cues.

**Supplemental Figure S3.** LS means ±SE on the untransformed scale. Females assayed in 2016 (N=92) paid more attention (seconds) to males than in 2017 (N=100).

## Tables and Table Legends

**Table 1.** Definition of behaviors

| Behavior | Description |
|---|---|
| In zone | When the focal female’s eye(s) crosses into the marked area located 6 cm in front of each male compartment. |
| In neutral zone | Female is not within 6 cm of any of the males; she is located in the 17cm x 31cm central (neutral) area located between the left and right-side male compartments. |
| Motionless | Female is not moving through water column for more than 2 seconds. |
| Swimming | Female is actively moving through water column. |
| Glass running | Female is swimming along the side(s) of the aquarium with her rostrum touching the glass (moving up and down the plastic divider was not included). |
| Orienting | Female turns her body such that she is pointing in the direction of the male. The female may be motionless or swimming directly toward a male. |

**Table 2.** Results of the GLMM for female attention directed toward males having one of three color pattern types: common, rare, or unfamiliar.

| **Effect** | **Num. DF** | **Den. DF** | **F** | **P-value** |
|---|---:|---:|---:|---:|
| **Male type** | **2** | **48.1** | **5.25** | **0.001** |
| **Male orange** | **1** | **70.5** | **5.61** | **0.021** |
| Male type * male orange | 2 | 46.5 | 2.82 | 0.070 |
| River | 5 | 22 | 1.34 | 0.284 |
| **Predation regime** | **1** | **23.2** | **6.90** | **0.015** |
| Male type * predation regime | 2 | 46.1 | 2.33 | 0.109 |
| Male orange * predation regime | 1 | 71.6 | 2.24 | 0.139 |
| **Male standard length (SL)** | **1** | **131** | **3.97** | **0.048** |
| SL * predation regime | 1 | 121 | 3.15 | 0.079 |
| **Year** | **1** | **16** | **23.60** | **<0.001** |
| SL * year | 1 | 110 | 2.74 | 0.101 |
| **Time in zone** | **1** | **530** | **908.09** | **<.0001** |

**Supplemental Table S1.** River name and predation regime, drainage, and GPS coordinates of populations sampled.

| River/predation regime | Drainage | Latitude – N | Longitude - W |
|---|---|---|---|
| Aripo high-predation | Caroni | 10.65474 | 61.22755 |
| Aripo low-predation (main river) | | 10.67123 | 61.22922 |
| El Cedro high-predation | Caroni | 10.6567 | 61.26599 |
| El Cedro low-predation | | 10.663588 | 61.26584 |
| Guanapo high-predation (Twin Bridges) | Caroni | 10.63989 | 61.24833 |
| Guanapo low-predation (Tumbasson) | | 10.70944 | 61.25778 |
| Marianne high-predation | Northern | 10.76667 | 61.30000 |
| Marianne low-predation | | 10.75727 | 61.31523 |
| Paria low-predation | Northern | 10.74740 | 61.26629 |
| Turure high-predation | Oropuche | 10.65469 | 61.16946 |
| Turure low-predation | | 10.68606 | 61.17312 |

**Supplemental Table S2.** River and predation regime, year sampled, pool identification, and number of females assayed from each pool. Marianne Downstream, Pool C (*) observations omitted due the death of the four males in this group.

| River | Year | Pool | Females Assayed |
|---|---|---|---|
| Aripo high-predation | 2016 | A | 5 |
| | | B | 4 |
| | | C | 5 |
| | | D | 3 |
| Aripo low-predation | 2016 | A | 7 |
| | | B | 7 |
| El Cedro high-predation | 2016 | A | 7 |
| | | B | 5 |
| | | C | 5 |
| El Cedro low-predation | 2016 | A | 6 |
| | | B | 7 |
| | | C | 6 |
| Guanapo high-predation | 2017 | AA | 6 |
| | | A | 6 |
| | | B | 7 |
| Guanapo low-predation | 2017 | A | 7 |

|  |  |  |  |
|---|---|---|---|
|  |  | B | 7 |
|  |  | C | 6 |
| Marianne high-predation | 2017 | B | 10 |
|  |  | C | 5* |
| Marianne low-predation | 2016 | A | 7 |
|  |  | B | 8 |
| Paria | 2016 | A | 5 |
|  |  | B | 5 |
|  | 2017 | A | 9 |
| Turure high-predation | 2017 | A | 7 |
|  |  | B | 6 |
|  |  | C | 7 |
| Turure low-predation | 2017 | AA | 8 |
|  |  | A | 8 |
|  |  | LS | 6 |

**Supplemental Table S3.** Quantitative comparison of male color pattern types. We computed pair-wise distance matrices between each type of male using two functions in R. We computed dissimilarity coefficients (A) using the *dist.binary* function in the *ade4* package. This function used the binary data (presence/absence) generated for each pixel coordinate of a specified color cluster (orange or black). We performed this analysis using two methods to calculate the dissimilarity coefficients (Method=1: Jaccard Index, Method=8: Sokal & Sneath). Both methods are for use on binary data; in contrast to the Sokal and Sneath method, the Jaccard Index does not include negative matches in the calculation, (Gower and Legendre 1986). We also evaluated the Euclidean distance (B) between individuals in PCA space for the orange and black color clusters using the *dist* function in base R. In the table, each column shows the mean and standard error for pairwise dissimilarity (or distance) for 11 populations used in the experiment. The three color pattern types of the four males are: common (C), rare (R), and unfamiliar (U).

| Measure | Color | C:C | C:R | C:U | R:U |
|---|---|---|---|---|---|
| (A) Jaccard Index | Orange | 0.921±0.048 | 0.957±0.016 | 0.956±0.016 | 0.960±0.027 |
| | Black | 0.868±0.044 | 0.893±0.049 | 0.896±0.045 | 0.900±0.043 |
| Sokal & Sneath | Orange | 0.865±0.076 | 0.919±0.024 | 0.919±0.025 | 0.926±0.046 |
| | Black | 0.784±0.055 | 0.821±0.067 | 0.825±0.060 | 0.831±0.063 |
| (B) Euclidean | Orange | 29.06±2.82 | 34.23±5.01 | 32.04±6.10 | 35.17±5.85 |
| | Black | 29.29±6.45 | 31.30±6.80 | 31.20±7.48 | 32.04±7.27 |

**Supplemental Table S4.** Parameter estimates from the mixed linear model.

| Effect | | Estimate | SE | DF | t Value | Pr >\|t\| |
|---|---|---|---|---|---|---|
| Intercept | | 0.24 | 0.16 | 61.7 | 1.81 | 0.075 |
| Male type | Common | -0.14 | 0.05 | 48.3 | -2.59 | 0.013 |
| | Rare | 0.00 | 0.06 | 71.9 | -0.05 | 0.958 |
| | Unfamiliar | 0 | . | . | . | . |
| Male orange | | 0.18 | 0.06 | 67.2 | 2.93 | 0.005 |
| Male type *male orange | Common | -0.06 | 0.04 | 45.1 | -1.45 | 0.153 |
| | Rare | -0.11 | 0.05 | 56 | -2.37 | 0.021 |
| | Unfamiliar | 0 | . | . | . | . |
| River | Turure | 0.22 | 0.15 | 24.8 | 1.48 | 0.151 |
| | Guanapo | 0.16 | 0.15 | 24.7 | 1.05 | 0.303 |
| | El Cedro | -0.003 | 0.14 | 32.9 | -0.02 | 0.985 |
| | Aripo | -0.01 | 0.14 | 29.8 | -0.09 | 0.932 |
| | Marianne | -0.09 | 0.15 | 36.1 | -0.62 | 0.540 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Paria | 0 | . | . | . | . |
| Predation regime | Low-predation (LP) | -0.14 | 0.08 | 52.4 | -1.85 | 0.069 |
| | High-predation (HP) | 0 | . | . | . | . |
| Male type * predation regime | Common*LP | 0.05 | 0.07 | 41.2 | 0.77 | 0.447 |
| | Common*HP | 0 | . | . | . | . |
| | Rare*LP | -0.11 | 0.08 | 64.4 | -1.33 | 0.187 |
| | Rare*HP | 0 | . | . | . | . |
| | Unfamiliar*LP | 0 | . | . | . | . |
| | Unfamiliar*HP | 0 | . | . | . | . |
| Male orange * predation regime | LP | 0.09 | 0.06 | 71.6 | -1.50 | 0.139 |
| | HP | 0 | . | . | . | . |
| Male standard length (SL) | | -0.04 | 0.05 | 146 | -0.74 | 0.461 |
| SL * predation regime | SL*LP | 0.09 | 0.05 | 121 | 1.77 | 0.079 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | SL*HP | 0 | . | . | . | . |
| Year | 2017 | -0.57 | 0.12 | 16 | -4.86 | <.001 |
| | 2016 | 0 | . | . | . | . |
| SL*year | 2017 | 0.09 | 0.05 | 110 | 1.66 | 0.101 |
| | 2016 | 0 | . | . | . | . |
| Time in zone | | 0.47 | 0.02 | 513 | 30.13 | <.0001 |

**Supplemental Table S5.** Post hoc pairwise comparison of female attention toward males having one of three color pattern types: common (C), rare (R), and unfamiliar (U). Differences in LS means estimates and standard errors are reported on the untransformed scale.

| Effect | Male type | Male type | Estimate | SE | DF | t Value | Unadj P | Adj P |
|---|---|---|---|---|---|---|---|---|
| Male type | C | R | -2.99 | 2.42 | 44.8 | -1.54 | 0.131 | 0.284 |
| **Male type** | **C** | **U** | **-5.08** | **2.27** | **42.6** | **-3.23** | **0.002** | **0.006** |
| Male type | R | U | -2.10 | 2.50 | 65 | -1.42 | 0.160 | 0.339 |

**Supplemental Table S6.** Results of the GLMM for female attention directed toward males having one of three color pattern types: unfamiliar, common, or rare. In this model, each population was given a unique identification.

| Effect | Num. DF | Den. DF | F | P-value |
|---|---|---|---|---|
| **Male type** | **2** | **30.9** | **4.20** | **0.024** |
| Male orange | 1 | 86.3 | 2.70 | 0.104 |
| Population | 10 | 17 | 2.17 | 0.076 |
| Male type * population | 20 | 28.1 | 1.04 | 0.455 |
| **Male standard length (SL)** | **1** | **193** | **4.31** | **0.039** |
| **Year** | **1** | **13.2** | **16.38** | **0.001** |
| **SL * year** | **1** | **192** | **5.22** | **0.024** |
| **Time in zone** | **1** | **487** | **869.88** | **<.0001** |

**Supplemental Table S7.** Parameter estimates from the mixed linear model where populations were given a unique identification. Predation regimes are indicated in the population name: High- and low-predation are represented by HP and LP, respectively. Common, rare and unfamiliar male color pattern types are represented by U, R, and C, respectively.

| Effect | Effect | Estimate | SE | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|---|
| Intercept | | 0.34 | 0.25 | 22.9 | 1.37 | 0.185 |
| Male type | C | -0.09 | 0.18 | 19.7 | -0.5 | 0.623 |
| | R | -0.04 | 0.19 | 23.8 | -0.21 | 0.837 |
| | U | 0 | . | . | . | . |
| Male orange | | 0.06 | 0.04 | 86.3 | 1.64 | 0.104 |
| Population | Turure LP | 0.18 | 0.20 | 29.9 | 0.87 | 0.391 |
| | Guanapo LP | -0.04 | 0.24 | 49.1 | -0.15 | 0.885 |
| | Turure HP | 0.23 | 0.20 | 31.2 | 1.13 | 0.266 |
| | El Cedro LP | -0.28 | 0.26 | 22.4 | -1.09 | 0.289 |
| | Paria | -0.17 | 0.23 | 24.4 | -0.75 | 0.458 |
| | Guanapo HP | 0.27 | 0.21 | 32.1 | 1.32 | 0.196 |

|  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|
|  |  | El Cedro HP | -0.23 | 0.26 | 23.3 | -0.87 | 0.394 |
|  |  | Aripo HP | -0.05 | 0.26 | 25.1 | -0.19 | 0.848 |
|  |  | Marianne LP | -0.30 | 0.27 | 22.5 | -1.13 | 0.269 |
|  |  | Aripo LP | -0.25 | 0.27 | 23.6 | -0.91 | 0.370 |
|  |  | Marianne HP | 0 | . | . | . | . |
| Population * male type | C | Turure LP | -0.05 | 0.22 | 22.6 | -0.23 | 0.818 |
|  | C | Guanapo LP | 0.02 | 0.21 | 21.9 | 0.09 | 0.927 |
|  | C | Turure HP | -0.16 | 0.22 | 23.8 | -0.75 | 0.461 |
|  | C | El Cedro LP | 0.15 | 0.21 | 20.3 | 0.74 | 0.469 |
|  | C | Paria | -0.06 | 0.21 | 21 | -0.3 | 0.769 |
|  | C | Guanapo HP | -0.04 | 0.22 | 23.6 | -0.17 | 0.869 |
|  | C | El Cedro HP | 0.05 | 0.21 | 22 | 0.25 | 0.807 |
|  | C | Aripo HP | 0.003 | 0.21 | 23.6 | 0.01 | 0.989 |
|  | C | Marianne LP | -0.07 | 0.23 | 20.6 | -0.3 | 0.771 |
|  | C | Aripo LP | -0.07 | 0.22 | 19.7 | -0.31 | 0.763 |
|  | C | Marianne HP | 0 | . | . | . | . |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| R | Turure LP | -0.24 | 0.23 | 27.8 | -1.08 | 0.291 |
| R | Guanapo LP | -0.04 | 0.24 | 33.2 | -0.15 | 0.880 |
| R | Turure HP | 0.07 | 0.23 | 28.6 | 0.32 | 0.754 |
| R | El Cedro LP | 0.26 | 0.22 | 27.4 | 1.15 | 0.258 |
| R | Paria | -0.17 | 0.22 | 27.8 | -0.75 | 0.458 |
| R | Guanapo HP | -0.10 | 0.23 | 30.6 | -0.42 | 0.679 |
| R | El Cedro HP | 0.12 | 0.23 | 28.9 | 0.54 | 0.596 |
| R | Aripo HP | 0.06 | 0.23 | 31.6 | 0.27 | 0.790 |
| R | Marianne LP | -0.02 | 0.23 | 25.2 | -0.1 | 0.923 |
| R | Aripo LP | -0.30 | 0.25 | 30.7 | -1.19 | 0.242 |
| R | Marianne HP | 0 | . | . | . | . |
| U | Turure LP | 0 | . | . | . | . |
| U | Guanapo LP | 0 | . | . | . | . |
| U | Turure HP | 0 | . | . | . | . |
| U | El Cedro LP | 0 | . | . | . | . |
| U | Paria | 0 | . | . | . | . |
| U | Guanapo HP | 0 | . | . | . | . |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | U | El Cedro HP | 0 | . | . | . | . |
| | U | Aripo HP | 0 | . | . | . | . |
| | U | Marianne LP | 0 | . | . | . | . |
| | U | Aripo LP | 0 | . | . | . | . |
| | U | Marianne HP | 0 | . | . | . | . |
| Male standard length (SL) | | | -0.01 | 0.04 | 161 | -0.16 | 0.873 |
| Year | | 2017 | -0.66 | 0.16 | 13.2 | -4.05 | 0.001 |
| | | 2016 | 0 | . | . | . | . |
| SL*year | | 2017 | 0.16 | 0.07 | 192 | 2.28 | 0.024 |
| | | 2016 | 0 | . | . | . | . |
| Time in zone | | | 0.47 | 0.02 | 487 | 29.49 | <.0001 |