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Original Article

Mating preferences do not maintain the tailspot polymorphism in the platyfish, *Xiphophorus variatus*

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Negative assortative, or disassortative, mating describes the scenario when individuals tend to mate with dissimilar phenotypes more often than by random chance alone. This form of nonrandom mating has the power to select for the maintenance of polymorphism in conspicuous traits, for example, when females prefer mates that have a different trait value than their own. The livebearing variable platyfish, *Xiphophorus variatus*, exhibits high polymorphism for a melanic pigmentation trait called tailspots, which results in variation in a conspicuous visual cue. Livebearers often exhibit female mating preferences based on morphological traits and disassortative mating has been suggested in a related species. Whether mating choice contributes to the maintenance of tailspot variation has never been tested. Here, we examined mating preferences of wild-caught females for pairs of male stimuli differing in their tailspot pattern. Females showed no evidence for either assortative or disassortative association preferences, spending equal time with males of different patterns. These results corroborate data on natural mating patterns in *X. variatus*, suggesting that mate choice for tailspots is either nonexistent or at least insufficient to account for the maintenance of tailspot variation.

Key words: association preference, disassortative mating, mate choice, melanism, pigment pattern.

INTRODUCTION

The maintenance of variation in conspicuous color patterns and the genetic polymorphisms that underlie them spans ecological (Shigemiyu 2004; Olendorf et al. 2006), physiological (Horth 2006; Price et al. 2008; Hegna et al. 2013), and behavioral (Houde 1987; Maffi et al. 2011) aspects of organismal biology. Female mate choice has long been recognized as an important source of selection on morphological variation (Andersson 1994). Evidence of the role of mating preferences and patterns in the maintenance of color and pattern polymorphisms is increasing (Houde 1987; Kingston et al. 2003; Takahashi and Hori 2008; Perez i de Lanuza et al. 2013). Specifically, positive or negative assortative mating in which females mate with similar or dissimilar individuals more frequently than expected by chance alone can promote the maintenance of polymorphism (Workman 1964; Scudo and Karlin 1969). Balanced polymorphism can also be produced through negative frequency-dependent selection in which rare individuals have a mating advantage (Farr 1977).

A number of studies documenting disassortative mating have accumulated in a variety of taxa (Emmel 1972; Takahashi and Hori 2008; Rolán-Alvarez et al. 2012). Such patterns are particularly common with visual signals such as differences in morphology including color and color pattern traits. Disassortative mating based on shell color contributes to maintenance of polymorphism in a species of intertidal snail in which males are choosy and search out female mates (Rolán-Alvarez et al. 2012). Roulin (2004) reviewed evidence for nonrandom mating preferences for color polymorphisms in birds and cited multiple species, which mate disassortatively with respect to plumage coloration. In the classic study system of color and pattern polymorphism in the guppy, *Poecilia reticulata*, females exhibit frequency-dependent patterns of mate preference, preferring rare and novel phenotypes (Farr 1977; Hughes et al. 1999). Those preferences likely promote the diversity of color and pattern combinations found in natural populations.

Though the study of coloration in fishes has a long history, the study of melanin-based traits and particularly sexual selection on these traits has received less focus (for review, see Price et al. 2008). The genus of livebearing fish *Xiphophorus* (Teleostei: Poeciliidae) has been widely studied in the context of female mate choice for

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morphological traits (Basolo 1990; Rosenthal and Evans 1998; Fisher et al. 2009; MacLaren et al. 2011) including color polymorphism (Kingston et al. 2003) and pigmented vertical bars (Morris and Casey 1998; Morris et al. 2006). Additionally, the spotted caudal (Sc) pattern of *Xiphophorus cortezi*, an irregularly shaped area of black pigment on the caudal peduncle and genetically linked to a functional oncogene, is preferred by females (Fernandez and Morris 2008). Furthermore, males with a malignant Sc pattern have larger body size, a trait preferred by *Xiphophorus* females, further conferring positive selection for the pattern and associated oncogene (Fernandez and Bowser 2010).

One of the most polymorphic patterns within species of the genus *Xiphophorus* is the micromelanophore tailspot pattern of the platyfish clade (Kallman 1975; Borowsky 1984). *Xiphophorus variatus* is a species of platyfish inhabiting streams of north- and east-central Mexico from the coastal plain to the foothills of the Sierra Madre Oriental. This species exhibits high polymorphism at the autosomal tailspot locus, with at least 6 known patterns that are exhibited in both sexes (Borowsky 1984). With the exception of one pattern that is controlled by a second modifying locus, the tailspot patterns of *X. variatus* are determined by alleles at a single autosomal locus, herein termed *Ts*. Pattern alleles are dominant over the wild type and codominant to one another. Wild-type fish have no pattern (i.e., no melanism on the caudal peduncle). Thus, at least 5 alleles are known in *X. variatus* and as many as 5 patterns occur together in the same population (Culumber et al. forthcoming). *Ts* exhibits pleiotropic effects on morphology and physiology. In addition to producing different pigment patterns at the juncture of the caudal peduncle and caudal fin, allelic variants exhibit differences in physiological tolerances (Borowsky 1984; Borowsky 1990; Culumber et al. forthcoming), and those studies have highlighted the importance of natural selection on the maintenance of the polymorphism. The extent to which sexual selection stemming from female mate choice may contribute to maintaining high polymorphism in this conspicuous trait has not been directly tested.

In a study on tailspot variation in the closely related *Xiphophorus maculatus*, Borowsky and Kallman (1976) reported evidence of disassortative mating patterns at the tailspot locus. Based on the tailspot phenotypes of females and their offspring, they inferred that females had mated with dissimilar males more often than expected by chance. When a similar study was conducted in *X. variatus*, there was no strong evidence of disassortative mating (Borowsky and Khouri 1976). As pointed out by Borowsky (1990), disassortative mating may exist in populations of *X. variatus* but be difficult to detect or rather to differentiate from random mating due to the frequencies of the tailspot patterns and potential distribution in their natural habitat. Furthermore, disassortative mating could exist in *X. variatus* but selection against heterozygotes, sperm competition, or genetic incompatibilities could result in mating patterns that appear random. As with ecological and physiological factors, it is important to test female mate choice in order to understand the extent to which it may contribute to the maintenance of the tailspot polymorphism.

Here, we examine female mating preferences in *X. variatus* to ascertain whether female mate choice matches patterns inferred from a natural population. Based on the limited data of tailspot parentage (Borowsky and Khouri 1976), we predicted that females would not discriminate among male tailspot types. We first focus on 2 allelic variants, wild type and cut crescent (Figure 1), testing female preference for a similar versus dissimilar type. Finally, to determine whether cue size (i.e., area of black pigment) might

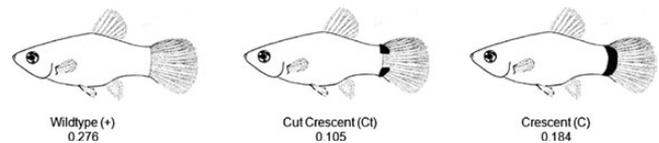


Figure 1

Illustrations modified from Gordon (1928) of the wild-type, cut crescent, and crescent tailspot types used in the present study. The frequency of each phenotype in the population at the time of collection is given below its name. One pattern found in the population was not used in this study, thus the frequencies do not sum to 1.

affect detectability and potentially limit response, we tested preferences between wild type and a larger pattern, crescent.

METHODS

Specimen collection and localities

Adult male and female *X. variatus* of 3 tailspot types were collected from the Río Atlapexco (GPS coordinates: 21.014, -98.339) in Hidalgo, Mexico. Figure 1 depicts the tailspot phenotypes that were used for this study and the frequency of all phenotypes in the population at the time of collection. All fish were transported to Texas A&M University where females were maintained in mixed-tailspot aquaria but were isolated from males for 1 month prior to mate choice trials. All fish were kept on a 12:12h light:dark cycle and fed the same diet, which consisted of TetraMin and spirulina flake foods.

Designing fish animations for playback

Mature males were digitally photographed for morphometric analysis. Male animations for video playback to females were created using the anyFish software platform (Veen et al. 2013). Briefly, 40 landmarks and semi-landmarks were placed on digital photographs of 6 males each of the wild-type, crescent, and cut crescent tailspots from the focal population using tpsDig2 software (Rohlf 2004). The landmark data from all males were then used to compute the consensus (population mean) morphology in tpsRwL (Rohlf 2007). A single digital image of a wild-type male *X. variatus* representing average coloration—no extreme yellows/oranges nor vertical bars—and having no tailspot pattern was then warped in tpsTransformer (Veen et al. 2013) in order to fit over the 3D model used by the anyFish software. The consensus morphology from the landmarked males was used inside anyFish to produce morphology representative of the mean male from the focal population. This base animation of a wild-type male was then used to create animations for each of the tailspot types. This was accomplished by using the software ImageJ (Abrámoff et al. 2004) to measure the size of each tailspot pattern on the same males used for landmarking. An equal-sized area of black “pigment” was then overlaid on the base animation at the juncture of the caudal peduncle and fin using Adobe Photoshop CS6 (Adobe Systems Incorporated). Animations consisted of the male swimming toward the middle of the screen, performing a “backward swim,” which is a common behavior when males are actively courting females, and then swimming offscreen. The animation then repeated in the opposite direction and this was looped continuously. The time for the fish to swim on-screen, perform a backward swim, and then swim offscreen was 15 s. We made every possible attempt to mimic the relative speed of males performing courtship in the wild. Males typically swim very fast

alongside a female until in front of her and then the backward swim is a slower movement that looks like the male just drifts backward for a few seconds a much slower speed than the forward swimming of the fish, but only last a short time. Thus, we mimicked these movements and relative speeds in the animation. The final size of the animation on the playback monitors was adjusted to match the mean size of the 18 males landmarked to generate the consensus shape of the animation (standard length = 31.6 mm). Thus, the animation of the wild-type, cut crescent, and crescent males was identical in every aspect except for the tailspot patterns. The background color was gray-blue (RGB: 200, 200, 200) and the substrate beige. The anyFish animation project files used to generate stimuli are archived on Dryad (<http://datadryad.org>; doi: 10.5061/dryad.6sg4q).

Mate preference testing

Preference testing closely followed published methods (Fisher et al. 2009; Verzijden and Rosenthal 2011). Preference tests were conducted in aquaria (51 × 28 × 33 cm) with a Samsung LCD monitor at each end to play video animations to females. A single female was introduced to each testing tank and allowed to acclimate for 10 min before video playback began. The playback procedure consisted of 300 s of black screen, 300 s of video playback of 2 animated stimuli, 300 s of black screen, and a final 300 s of video playback with the same 2 animated stimuli switched to opposite monitors. In this way, we could control for side bias within each trial. The side on which a stimulus was first shown was randomized across trials and tanks.

We first conducted a control experiment to determine whether females were responsive to the male animations. For this test, a random selection of females were given a choice between the mean male animation (no tailspot pattern) and the standard animation backdrop with no fish to ensure that females were responsive to the animated fish. These females were not used in subsequent trials. Next, to test for nonrandom mating preferences with respect to tailspot pattern, we first tested wild-type and cut crescent female preferences for pairs of wild-type versus cut crescent-patterned stimuli. Based on the outcome of those trials, and in order to determine whether tailspot pattern size or visibility affected preferences, we then tested crescent females for preferences between wild type and crescent. The crescent pattern—extending from the top to the bottom of the caudal peduncle—has greater area of pigment than the cut crescent pattern and therefore produces a larger signal that may be more easily detected. Finally, we conducted a positive control test to show that females were motivated to attend to differences in male stimuli. A previous study in *X. variatus* showed that females attended to male dorsal fin size in the context of lateral projection area (MacLaren et al. 2011). We, therefore, tested a random subset of wild-type and cut crescent females with 2 animated males that differed only in dorsal fin size. This comparison was between the mean male animation and the mean animation in which the dorsal fin area was doubled. The result was an animated stimulus of the same standard length, but for which the lateral projection area was larger for the male with the enlarged dorsal fin. We predicted that females would attend to differences in dorsal fin size of the male stimuli and this was considered a positive control to test that females were motivated to respond to differences in stimuli such as the tailspots of the focal association trials.

All preference trials were video recorded and videos were used to score association time of females with animated stimuli. All videos were scored blindly without knowing what stimulus was on which

side until all videos had been scored. To determine association time, tanks were divided into 3 equally sized zones by placing small marks on the edge of the test aquaria that were visible only to the human observer. Females were considered to be associating with a stimulus anytime they were in the zone nearest that stimulus. If a female's body spanned the boundary between zones, she was only considered to be associating with the stimulus if she was oriented toward (i.e., facing) that stimulus. Association time was recorded by watching videos of the trials and using stopwatches to time spent in the association zones. Time spent in the center zone was not counted. Association time is a standard metric of mate preference in *Xiphophorus* and has been shown to be a strong and repeatable predictor of mate choice and reproductive outcomes (Cummings and Mollaghan 2006; Walling et al. 2010). We calculated each female's mean association time with each stimulus by averaging across the 2 playback periods. Paired *t*-tests were used to compare mean association times between stimuli. A Pearson correlation was calculated to test for repeatability of female preferences between the 2 playback periods.

RESULTS

Females responded positively to the fish animation ($t_9 = 4.88$, $P = 0.0008$; Figure 2A) spending an average of 194 ± 14.2 s (\pm SE) with the fish compared with only 73 s (± 11.1 s) with the background without a fish. Females also exhibited a significant preference between males differing in dorsal fin size ($t_{19} = 2.19$, $P = 0.04$). Mean association time with the small dorsal fin male was 160 s (± 13.8 s) and 103 s (13.0 s) with the large dorsal fin. However, there was no evidence of either positive or negative assortative mate preference in any of the tested tailspot types (Figure 2B). Neither wild-type nor cut crescent females exhibited a preference between the wild-type and cut crescent pattern. Wild-type females spent 140 s (± 9.3 s) with the wild-type male and 134 s (± 9.4 s) with the cut crescent ($t_{22} = 0.358$, $P = 0.72$; Figure 2B). Similarly, cut crescent females spent 144 s (± 13.2 s) with wild-type and 129 s (± 13.6 s) with the cut crescent male ($t_{18} = 0.584$, $P = 0.567$; Figure 2B). The crescent pattern—approximately 2.6 times larger in area than the cut crescent pattern—was used to test whether the cut crescent pattern might be too small for females to reliably detect due to low signal size. There was no effect when crescent females were given a choice between wild-type and crescent animations ($t_{11} = 0.00852$, $P = 0.99$; Figure 2B). Crescent females spent a mean of 130 s (± 14.3 s) with the wild-type animation and 131 s (± 16.4 s) with the crescent. Across all females, there was no correlation between female preferences in the first playback period and preference in the second playback period ($r = -0.263$, $N = 44$, $P = 0.084$). Preferences between playback periods were also not correlated when the analysis was restricted within female tailspot type (data not shown).

DISCUSSION

There was no evidence of either positive or negative disassortative mating preferences for tailspots, or of negative frequency-dependent selection for rarer morphs. Female *X. variatus* exhibited random preferences, spending equal time with males of similar and dissimilar tailspot types. These findings are consistent with field data on mating patterns suggesting that *X. variatus* females mate randomly with respect to tailspot pattern. Given the potential for postcopulatory selection to modify mate choice, our findings

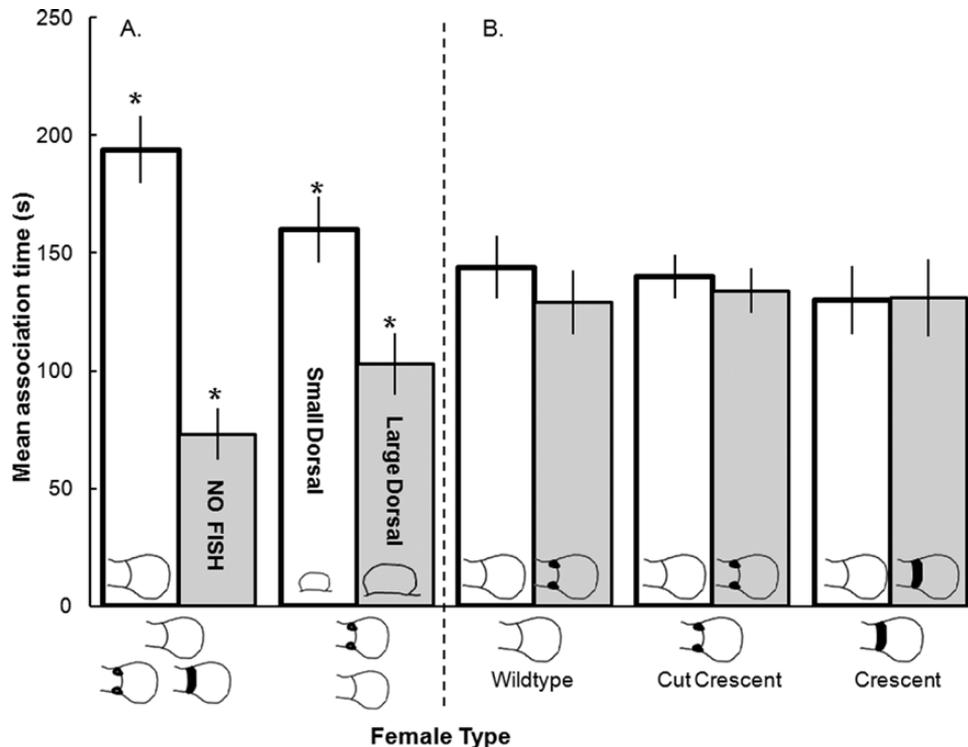


Figure 2

(A) Mean association times (\pm SE) of *Xiphophorus variatus* females in control experiments to determine responsiveness to the male animation and ability to choose between 2 male stimuli. A random sample of females was used for both tests. In both tests, females exhibited significant differences in association times between stimuli, denoted by asterisks ($P < 0.05$). (B) There was no evidence of either positive or negative assortative preferences of females for tailspot patterns. Regardless of tailspot type, females spent equal time with both male stimuli. The images below the x axis represent the female tailspot types that were tested in each associated experiment.

demonstrate that mating outcomes are consistent with mating preferences. The results of our study contrast with findings in *X. maculatus* that show disassortative mating patterns, which suggests that, in *X. variatus*, natural selection may be the primary driver of the maintenance of the tailspot polymorphism. There was no evidence that tailspot pattern size (pigment area) is a limiting factor in eliciting female response.

Females responded positively in both control experiments, demonstrating their propensity to respond to the fish animation and to choose between pairs of male stimuli. In a previous study, female *X. variatus* preferred males of larger lateral projection area (i.e., larger dorsal fin for a fixed body size; MacLaren et al. 2011). Here, females preferred a smaller dorsal fin when body size was held constant. A number of factors could account for preference for smaller dorsal fins including recent exposure (cf., Verzijden and Rosenthal 2011) because females had been exposed to the male with the smaller dorsal fin during prior trials. Within the context of this study, however, the critical finding is that females did show a significant preference for one male over the other, demonstrating ability to attend to visual signals and choose between stimuli.

Previous investigation of mating patterns in *X. variatus* involved collecting gravid females from the wild and determining the tailspot phenotype of their offspring. This study suggested no deviation from random mating and, specifically, no evidence of disassortative mating (Borowsky and Khouri 1976). Our findings contrasted with a similar study first conducted on *X. maculatus*, which demonstrated disassortative patterns (Borowsky and Kallman 1976). Females of the wild-type, cut crescent, and crescent tailspot

types exhibited random preferences in mate choice trials, spending equal time with a male of their own tailspot type as they did with a male of a dissimilar type. It seems unlikely that nonrandom mating preferences contribute to the maintenance of the tailspot polymorphism in a meaningful way for *X. variatus*. However, one possibility is that mating and mate preferences may be frequency dependent. There is ample evidence, including examples in poeciliids, that early experience and recent exposure can alter female preference (Verzijden and Rosenthal 2011; Verzijden et al. 2012). For example, exposure to heterospecifics can either strengthen or weaken female preferences for conspecifics or even generate preference in a new direction (e.g., toward heterospecifics; Verzijden et al. 2012). The frequency of phenotypes in a population can also affect female choice, and negative frequency-dependent selection could be a mechanism favoring balanced polymorphism at the tailspot locus. Examples of negative frequency-dependent selection have been reported in 2 closely related species (Hughes et al. 1999; Royle et al. 2008). Such patterns of mating and mate preferences could be difficult to detect in *X. variatus*, because allele and pattern frequencies can change from one generation to the next owing to selection from environmental variables like temperature (Culumber et al. forthcoming).

The difference in mating patterns in natural populations between *X. variatus* and *X. maculatus* observed in the 1976 studies is puzzling. However, low sample size could make it difficult to detect such patterns. As previously pointed out, large sample sizes are likely necessary to detect nonrandom mating in *X. variatus* (Borowsky 1990). The sample of females for mating pattern inference in Borowsky

and Khouri (1976) consisted of a mean of 14 females per pool over 4 pools. Based on the frequency of individuals in that population, the author later suggested that a sample on the order of >1000 individuals may be necessary to reliably detect nonrandom mating based on tailspot patterns of offspring (Borowsky 1990). However, with a comparatively much smaller sample size, we are able to more robustly test mating preferences. For the population and frequency of tailspot phenotypes sampled in the present study, neither positive nor negative assortative mating was observed. This suggests that, regardless of the power of the 1976 study to detect deviations, assortative mating for T_s is unlikely in *X. variatus*. There was also no evidence of negative frequency-dependent preferences; females did not exhibit preferences for the rarest tailspot types (crescent and cut crescent). Frequencies of female tailspot types experienced by females during isolation prior to experiments were likely not identical to the natural population. Therefore, future work will be necessary to further investigate the potential for frequency-dependent choice arising from variation in the frequency of phenotypes in natural populations.

Alternatively, females may simply not attend to tailspot phenotype as a part of mate choice or only in certain contexts. Tailspots may be important in mate choice or mate recognition only during times when the visual environment is degraded. For example, Franck et al. (2001) observed that female *Xiphophorus hellerii* exhibited preferences for males with macromelanophores—large spots of melanic pigment on the body—only in turbid water where melanism increases detection, but females showed no preference in clear water. A similar pattern may exist for the tailspot trait. Female *X. variatus* may not attend to tailspot patterns in clear water, but males with patterns may be more easily detected and have a mating advantage during the rainy season when the water is turbid. Physiological effects of alleles at the tailspot locus have also been implicated in the maintenance of the polymorphism. For example, fish of different patterns have different physiological thresholds for dissolved oxygen and temperature, which likely plays an important role in the maintenance of tailspot variation (Borowsky 1990; Culumber et al. forthcoming).

Mating patterns in a natural population and now mate preference data from a separate population suggest that nonrandom mating with respect to tailspot pattern does not play a noticeable role in the maintenance of the tailspot polymorphism in *X. variatus*. Though female mate choice has been shown to be an important source of selection for color polymorphism in related *P. reticulata*, this does not appear to be the case with tailspots of the platyfish in the genus *Xiphophorus*. Our findings lend further support to the argument that environmental variation is the primary factor affecting tailspot variation. However, given the potential for experience or phenotypic frequencies to affect female preferences and for postcopulatory selection to obscure mating patterns, future studies will be able to continue to disentangle the relative contributions of natural and sexual selection to the maintenance of this conspicuous polymorphism.

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