**Habituation underpins preference for mates with novel phenotypes in the guppy**

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**Supplementary Materials**

**Supplementary Methods**

*Husbandry*

The males and females used in our experiment were placed in rearing tanks within 12 h after birth. These tanks were visually isolated from tanks containing adult males so that females would remain naïve to male colour patterns. As they matured, we transferred fish into single-sex tanks where they were held until they were used in the experiment. Females in these holding tanks were also visually isolated from any adult males. Our fish tanks were lined with a layer of natural coloured gravel on the bottom, and illuminated by both incandescent and full spectrum fluorescent bulbs held approximately 30 cm above the water’s surface. Fish rooms were kept on a 12:12 h light:dark cycle, at 25-26° C. We fed our fish twice daily: Tetramin flake food in the mornings, and live *A.* *nauplii* in the afternoons.

*Origin and maintenance of Iso-Y lines*

The males used in our experiment were derived from two Iso-Y lines (lines 9 and 10; Figure 1). These lines were originally created by AE Houde, who established them by choosing male lineages in which body colour patterns were highly heritable and Y-linked. Consequently, males from the same line have similar colour patterns, and males from different lines have different colour patterns. Because these lines were drawn from natural colour pattern variation, the patterns (and their level of heritability) are ecologically relevant. These lines have been maintained at Florida State University since 2012 by mating males from each line to randomly selected females derived from the same natural population as the males used to found these lines (Paria Tributary, Trinidad).

*Stimulus exposure*

We took several measures to ensure that females were attending to male colour patterns consistently when they were exposed to males as part of our habituation paradigm. Females were exposed to males in a habituation tank consisting of a male and female compartment (each 15 x 20 x 15 cm), separated by internal dividers spanning 5 cm. This was done by placing a male in the male compartment, while the opaque divider was lowered, and then raising the divider to allow the fish to interact across the clear dividers. After the two-minute exposure period, the opaque divider was lowered, and the male was replaced with a different male from the same IF line. After one minute, the door was raised again for a second exposure period. This process was repeated following the habituation paradigm outlined in the main text. When guppies are allowed to freely interact, males are typically persistent in their pursuit of females, and move among females on a timescale of minutes. This means that the timeframe of our exposure periods was ecologically relevant. Furthermore, previous work has shown that exposure to male colour patterns can induce changes in female preference over this timescale [1].

The clear dividers on either side of the opaque divider prevented males and females from mating, as changes in a female’s reproductive status could have biased her subsequent mating interest. These clear dividers were watertight, ensuring that any effects of exposure to males was attributable to visual cues and not chemosensory communication. This controlled for any potential differences in olfactory cues between lines. Moreover, we found no evidence that the Iso-Y lines used in our experiment differed in their sexual behaviours (see results). Therefore, effects of exposure to stimulus males on female mating interest can be attributed to the appearance of the stimulus males.

By netting males into and out of the tank behind the opaque divider, we minimized disturbance to females. Furthermore, the pulley and lever system was operated out of view of the fish, and females and males were acclimated to movement of the opaque divider prior to being used in the experiment. This was important because it prevented stress responses that would have distracted females from the male, such as the dart and freeze behaviours that guppies typically exhibit in response to the introduction of a net to the tank. To ensure that males and females did not exhibit these dart and freeze behaviours in response to movement of the opaque divider, we acclimated fish to movement of the divider prior to conducting the experiment. This was done by placing fish individually in the habituation tank and repeatedly raising and lowering the divider (following the same 2:1 min schedule as used in the experiment). Fish were exposed to the divider’s movement 15 times per day on consecutive days until they did not dart or freeze in response to the dividers movement on the first 4 movements of the divider that day. This took an average of 4.32 ± 0.73 (mean ± SE) days of training. A small subset of fish (n = 7 females; n = 2 males) that had not stopped these responses by the end of 6 days of training were excluded from the study.

Prior to conducting our experiment, we screened males based on the level of mating effort that they exhibited when allowed to interact with (non-experimental) females. We selected males that showed relatively high levels of sexual behaviours, as these males should be more likely to solicit the attention of the females during the exposure periods (ensuring females consistently attended to the males), and during the mating trials (ensuring that we would have ample behavioural interactions from which to infer female mating interest). We did this by placing each male with a single female in the observation tank, and scoring his sexual behaviours for 5 min. Only males that courted the female at least 3 times within this timeframe were used in our experiment (50/89, 56%).

*Mating interest assays*

We measured female mating interest by placing the female in an observation tank (40 x 20 x 20 cm) and, after a 1 min acclimation period, adding a test male. The two fish were allowed to freely interact for 5 min, while their reproductive behaviours were scored manually. Male guppies are nearly incessant in their pursuit of matings, and flexibly exhibit two mating strategies: sneak mating attempts, and courtship displays that advertise their colour patterns to females. Female guppies are choosy and most often ignore male courtship [2]. When interested in a male, a female can respond positively by engaging in several types of positive response behaviours (see Supplementary Table 1 for ethogram). These positive female responses are sometimes followed by cooperative copulations, and are widely used as a measure of female mating interest [2].

Females were all virgins at the start of the experiment, in order to avoid variation in mating history or exposure to males that could have affected mating interest. While female guppies are choosier once mated [3], virgins do show strong preferences for rare and novel colour patterns (e.g. [4,5]). Guppies are most sexually active in the mornings. Consequently, we performed all stimulus male exposures and behavioural observations between 9 AM and noon to minimize diel variation in reproductive behaviours. Our sampling design was counter-balanced with respect to all possible combinations of treatment, the Iso-Y line of the stimulus males, and the time of testing. These combinations of factors were as evenly distributed among days of testing as possible, given the constraint of testing 10 females per day (see Supplementary Data). We used a total of 50 males (25 per line), and used males with multiple females. However, a female was never assigned the same male more than once throughout exposure and testing. This decoupled habituation to colour patterns from familiarity with individual males. We also did not use the same male(s) with back-to-back females to avoid male fatigue. The water in the exposure and mating trial tanks was replaced at the end of each day to prevent olfactory carry-over.

**Supplementary results**

In the main text, we analysed female mating interest as the proportion of a male’s courtship displays to which the female responded positively. All model estimates and test statistics are provided in Supplementary Table 2. We also provide a point of comparison for this metric by repeating our analyses with another commonly used measure of female mating preference in this species: number of positive responses, which is a count of the number of times a female responded positively to her suitor. We fit generalized linear mixed models following the same statistical approach outlined in the text, but used a Poisson distribution. As detailed below, analyses of number of positive responses revealed results that are qualitatively equivalent to those reported in the main text.

Females responded to male courtship an average of 2.709 ± 0.115 times per trial (mean ± SE). The interaction between treatment and line was not significant (= 12.592, *P* = 0.247), and was therefore excluded from the final model. Female mating interest was significantly influenced by both treatment ( = 50.361, *P* < 0.001) and line ( = 0.168 ± 0.082, = 4.200, *P* = 0.040). As in the main text, these results indicate (i) an effect of visual exposure to males, (ii) a directional preference for line 9 over line 10, and (iii) no evidence that habituation differed between lines.

We again found evidence of responsiveness decline (naïve vs. 15 treatments: = 0.598 ± 0.188, = 3.185, *P* = 0.001), spontaneous recovery (15 vs. SR treatments: = 0.479 ± 0.192, = 2.494, *P* = 0.013), dishabituation (15 vs. Dis treatments: = 0.464 ± 0.192, = 2.413, *P* = 0.016), and stimulus specificity (15 vs. SS treatments: = 0.343 ± 0.197, = 2.140, *P* = 0.032). We also similarly found evidence for long-term responsiveness decline ( = 0.417 ± 0.239, = 2.143, *P* = 0.032) and long-term stimulus specificity ( = 0.545 ± 0.233, = 2.334, *P* = 0.020). As in the main text, post-hoc tests indicated that exposure to 6 males with a given colour pattern was sufficient to elicit a decline in female mating interest in males with that same pattern (Supplementary Table 3). Collectively, these results indicate that our results are robust to the particular method used to quantify female mating interest.

To shed light on the overall preference we observed for males from line 9 over line 10, we characterized the amount of orange coloration and standard length of 25 males from each line. These data met parametric assumptions, so we compared the two lines using unpaired t-tests. Males from line 9 had significantly more orange coloration, measured as a proportion of their total body area (line 9: 14.453 ± 1.06 %; line 10: 10.535 ± 0.817 %; t = 2.923; df = 48; *P* = 0.005). The lines did not differ in standard length (line 9: 15.687 ± 0.250 mm; line 10: 15.240 ± 0.287 mm; unpaired t-test: t = 1.176; df = 48; *P* = 0.245). Given that female guppies from our study population have been shown to prefer males with more orange coloration [2,11], the greater amount of orange on line 9 males may explain the overall preference for males from that line.

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Supplementary table 1. Ethogram of guppy reproductive behaviours (following [2,6])

|  |  |  |
| --- | --- | --- |
| Behaviour | Criteria | Biological interpretation |
| Sigmoid display | Male shows his side to the female and quivers with an arched body | Courtship |
| Female positive responses    Orient | In response to sigmoid displays, female:    Turns to face male | Indicators of female interest (ordered from least to most intense) |
| Approach | Moves toward male |  |
| Glide | Uses pectoral fins to drift  directly in front of the  male |  |
| Cooperative copulation | Female and male swim together in swift, circling motion | Mating |

Supplementary table 2. Estimates and test statistics from the generalized linear mixed model analysing female mating interest. Effects are reported on the transformed scale.

|  |  |  |  |
| --- | --- | --- | --- |
| Fixed effects | ± SE | z value | P value |
| Intercept | -0.276 ± 0.151 | -1.823 | 0.068 |
| Line 10 | -0.441 ± 0.524 | -2.407 | 0.016 |
| 3 | -0.275 ± 0.211 | -1.306 | 0.192 |
| 6 | -0.832 ± 0.224 | -3.721 | <0.001 |
| 9 | -0.939 ± 0.222 | -4.227 | <0.001 |
| 12 | -1.139 ± 0.237 | -4.814 | <0.001 |
| 15 | -0.944 ± 0.223 | -4.227 | <0.001 |
| Spontaneous recovery | -0.312 ± 0.206 | -1.516 | 0.129 |
| Dishabituation | -0.320 ± 0.206 | -1.552 | 0.121 |
| Stimulus specificity | -0.352 ± 0.212 | -1.659 | 0.097 |
| Long-term responsiveness decline | -1.456 ± 0.247 | -5.888 | <0.001 |
| Long-term stimulus specificity | -0.531 ± 0.222 | -2.393 | 0.017 |
| Random effects | Variance\* | Stdev\* |  |
| Day of testing | 0 | 0 |  |
| Time of testing | 0 | 0 |  |

\*The lme4 package can produce variance estimates of 0 when the true variance is equal to 0 or is very small. This is because the sampling distribution of the estimate consists of a point mass at 0 and chi-squared distribution away from zero, which allows the log-likelihood to be maximized at 0 [7,8].

Supplementary Table 3. Results of post-hoc tests comparing number of positive responses between naïve females and females exposed to 3, 6, 9, or 12 stimulus males. Significant *P* values are bolded.

|  |  |  |  |
| --- | --- | --- | --- |
| Comparison |  |  | *P* |
| Naïve vs. 3 | 0.208 ± 0.167 | 1.243 | 0.214 |
| Naïve vs. 6 | 0.575 ± 0.186 | 3.088 | **0.006** |
| Naïve vs. 9 | 0.575 ± 0.187 | 3.087 | **0.006** |
| Naïve vs. 12 | 0.827 ± 0.203 | 4.079 | **< 0.001** |

 Supplementary Figure 1. Hypothetical mating interest for females from each experimental treatment. We tested predictions associated with the following four criteria of short-term habituation, which are widely used to distinguish between short-term habituation and alternative explanations [9,10]. To look for evidence of responsiveness decline, we tested the prediction that the mating interest of females in the 15 treatment should be lower than in the naïve treatment. Comparing the naïve treatment against treatments 3, 6, 9, and 12 allowed us to also ask how many exposures were required to elicit responsiveness decline. If spontaneous recovery occurs, we predicted that the mating interest of females in the spontaneous recovery (SR) treatment should be greater than females in the 15 treatment. We tested for dishabituation by asking whether females in the dishabituation (Dis) treatment showed greater interest than those in the 15 treatment. As a test of stimulus specificity, we asked whether females in the stimulus specificity (SS) treatment showed greater mating interest than those in the 15 treatment. We additionally looked for evidence of long-term habituation by testing the following predictions. If long-term responsiveness decline occurred, we expected that the mating interest of females in the long-term responsiveness decline (LTRD) treatment should be lower than in the naïve treatment. Finally, if there is long-term stimulus specificity, females in the long-term stimulus specificity (LTSS) treatment should show lower mating interest than females in the LTRD treatment.