



# The role of sexual selection in maintaining a colour polymorphism in the pygmy swordtail, *Xiphophorus pygmaeus*

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The forces responsible for maintaining genetic polymorphisms within populations are often unclear. Male pygmy swordtails are polymorphic at a single Y-linked locus that regulates body colour. The visually conspicuous, sex-limited polymorphism suggests that sexual selection is important for maintaining genetic variation for these conspicuous phenotypes. We performed a series of behavioural experiments that decoupled body colour from possible correlates, to detect female mating preferences for each morph and differences in male reproductive behaviour. Male body colour, male mating behaviour and male agonistic behaviour all appeared to influence male reproductive success. Females in two of three populations showed mating preferences for blue males, but gold males dominated in agonistic interactions and were more aggressive in pursuing females. Females in those populations preferred blue to gold males under optical filters that masked blue colour, but not under filters that masked gold colour, suggesting that female behaviour reflects an aversion to gold males. Females from one population characterized by high predation risk, the Nacimiento of the Río Huichihuayán, Mexico, showed no mating preferences for blue males, a significantly different result from those for females from two populations with a lower predation risk. Both female mating preferences and male competitive behaviour thus appeared to be important for maintaining the colour polymorphism. The lack of preference in the Nacimiento population is consistent with previous studies showing reduced preferences for conspicuous traits in this population.

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In the competition for mates, there must be both winners and losers. Those who are disadvantaged under one set of conditions often resort to alternative tactics to secure matings, and thus many mating systems are characterized by multiple strategies for procuring mates (Andersson 1994). Alternative mating strategies are often viewed as single entities, with little attention paid to the different behavioural and morphological factors that comprise the strategy (Dominey 1980; Gross 1985; Shuster & Wade 1991; Lank et al. 1995). Most alternative strategies, however, comprise a suite of traits, and behavioural differences in mating strategies are often accompanied by substantial differences in morphology (Andersson 1994). Polychromatism, in which there are discrete individual differences in coloration, represents a particularly striking form of morphological differentiation. Despite the

ubiquity of polychromatism in nature (Barlow 1973; Ryan & Keddy-Hector 1992; Andersson 1994; Hoffman & Blouin 2000), only a handful of studies have addressed the role of sexual selection in maintaining colour differences in a population (Barlow 1983a, b; Baer et al. 1995; Ellingson et al. 1995).

The two sexual selection mechanisms described by Darwin (1871), mate choice and competition for mates, can act to maintain morphological variation, such as colour, within a species. For example, most Midas cichlids, *Theraps* ('*Cichlasoma*') *citrinellum*, are grey but a small proportion are gold (Barlow 1973). Although gold morphs make up only a small percentage of *T. citrinellum* populations (on average <10%), they are found in most populations of the species, suggesting a balanced polymorphism (Barlow 1983a). Studies examining male and female reproductive behaviour and population frequency suggest that both female preference and male competition maintain variation within populations. Gold *T. citrinellum* are more aggressive (McKaye & Barlow 1976), and coloration appears to confer an advantage in agonistic interactions (Barlow 1983b). Any

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countervailing advantage of grey coloration, however, is not clear, although Barlow (1983a) suggested that gold morphs might be more conspicuous to predators. Both males and females are polymorphic, and tend to mate assortatively with respect to morph (Barlow & Rogers 1978). Similarly, Lake Victoria cichlids (genus *Haplochromis*) also mate assortatively with respect to colour, which may partially account for rapid speciation in these taxa (Seehausen & van Alphen 1998) but does not account for the maintenance of the balanced colour polymorphism among conspecifics.

Coloration in *Haplochromis* and *Theraps* thus appears to be selectively neutral with respect to mate choice. In contrast, in the gecko *Gonatodes albogularis*, in which male head coloration varies from reddish-orange to yellow-green, yellow-headed males are more conspicuous to females (Ellingson et al. 1995), more likely to mate, and more likely to win aggressive encounters, even when matched for size. These advantages are offset by increased predation risk for yellower males (Ellingson 1994). Yellow males may thus hold a mating advantage over other males, but only if they survive to reproductive age. A trade-off between survivorship and mating success could be responsible for maintaining variation in equilibrium (Ryan et al. 1992). In the present study, we sought to identify factors maintaining a dramatic colour polymorphism in a poeciliid fish, *Xiphophorus pygmaeus*, where a single Y-linked factor is correlated with a difference in male colour.

## Background

The northern, or Río Pánuco basin, swordtails (Pisces: Poeciliidae, genus *Xiphophorus*) are a monophyletic group of nine species endemic to the eastern slopes of Mexico's Sierra Madre Oriental (Rauchenberger et al. 1990; Marcus & McCune 1999; Morris et al. 2001). In *X. multilineatus* and *X. nigrensis*, most variation in male body size can be explained by variation at the Y-linked *P* locus (reviewed in Kallman 1989). Small males reach sexual maturity earlier, are slender-bodied, and lack conspicuous courtship displays or sexual ornamentation. Large and intermediate males mature later, have robust, ornamented bodies, and perform elaborate displays to potential mates (Ryan & Causey 1989; Zimmerer & Kallman 1989). Females prefer to mate with larger, courting males (Zimmerer & Kallman 1989; Ryan et al. 1990). The larger males' advantage with respect to sexual selection is offset by natural selection: larger males are less likely to reach sexual maturity (Ryan et al. 1992) and are more conspicuous to predators (Rosenthal et al. 2001a).

A second Y-linked polymorphism, with three alleles at a single locus, underlies colour variation. Males carrying the *+* and *cp* alleles express blue body colour, with *cp* males expressing a gold caudal fin. *Con* males have both a gold body and caudal fin (Kallman 1989; Baer et al. 1995). The *con* allele occurs only in small males, while the *+* and *cp* alleles occur in males of all size classes (Kallman 1989).

In *X. pygmaeus*, males are fixed for the small, noncourtship allele at the *P* locus, but males are polymorphic for body colour; 13–25% of small males are gold (Baer et al.

**Table 1.** Frequencies (%) of male colour morphs in three *X. pygmaeus* populations

Population	N	Blue males		Gold males
		<i>+</i>	<i>cp</i>	<i>con</i>
Nacimiento Huichihuayán	53	53	28	19
Huichihuayán Town	51	73	2	25
la Y-griega Vieja	52	69	17	13

1995; Table 1). In the closely related *X. nigrensis* and *X. multilineatus*, about one in 10 small males carries the *con* allele (G. G. Rosenthal & M. J. Ryan, unpublished data), a substantially lower percentage relative to *X. pygmaeus* (Table 1). The extreme colour difference between blue and gold males (Fig. 1), along with sexual dimorphism in body colour, suggests that sexual selection might be important for maintaining the polymorphism. Furthermore, colour may be correlated with behavioural traits involved in both male–female and male–male interactions. In Midas cichlids, for example, gold cichlids are dominant in aggressive interactions with wild-type individuals (McKaye & Barlow 1976; Barlow et al. 1977; Barlow 1983b).

We evaluated whether female mating preferences in *X. pygmaeus* were acting on male colour, and whether male colour traits were associated with behaviour in intra- and intersexual interactions. Another critical question is how populations vary in their response to gold and blue males. In *X. pygmaeus*, three populations on the Río Huichihuayán vary substantially in their preferences for large body size (Morris et al. 1996) and for male ornaments, courtship and conspecifics, possibly as a consequence of differences in predation risk (Rosenthal 2000a). The frequency of entirely blue (*+*) males is markedly lower at the Nacimiento of the Río Huichihuayán (Table 1), suggesting that colour preferences may vary as well.

## METHODS

We used a series of five experiments, in which we decoupled male body colour from behaviour, to detect female mating preferences and differences in male reproductive behaviour. We conducted 'free-range' experiments allowing direct female–male interactions to investigate the direct effects of male behaviour on mating success. Dyadic interactions (Morris et al. 1992) between randomly selected, size-matched pairs of blue and gold males were used to assess the outcome of blue–gold agonistic interactions. The preference stimuli included both live males and monochromatic digital animations with colour filters. Female preference for blue or gold males was tested with live males. We used colour filters (Custom Scientific) approximately matching the spectral reflectance functions of blue and gold male body coloration (Fig. 1) to test female preference for male body colour. We also used filters that masked colour variation in live males (Milinski & Bakker 1990), to test female preference for male behaviour. Table 2 summarizes the experiments.

**Table 2.** Summary of experiments

Experiment	Traits held constant	Examines
Male–female direct interaction	Male body colour, size and shape	Male mating behaviour
Male–male direct interaction	Male body colour, size and shape	Male competitive behaviour
Live stimuli	Male body size and shape	Female preference for colour and behaviour
Video animation	Male body colour, size, shape and behaviour	Female preference for colour
Live stimuli with colour filters	Male body colour, size and shape	Female preference for behaviour

We obtained fish from stocks maintained in large (4000-litre) outdoor pools at the Brackenridge Field Laboratory at the University of Texas at Austin, U.S.A. All test subjects were descendants of individuals collected, with permission of the Government of Mexico, between 1994 and 1999 from three localities, Nacimiento Huichihuayán (21°27'34.1"N, 98°58'36.7"W), Huichihuayán Town (21°28'48.1"N, 98°58'0"W) and la Y-griega Vieja (21°27'8.8"N, 98°56'18.8"W), all on the Río Huichihuayán, San Luis Potosí, Mexico (Morris et al. 1996). Experiments were carried out between April 1999 and March 2000 and we used only sexually mature individuals familiar with conspecific males of both morphs and with females.

Test subjects were maintained in the laboratory on a 14:10 h light:dark cycle with both fluorescent and natural light provided during daylight hours. They were fed daily TetraMin flake food occasionally supplemented by live brine shrimp and bloodworms. We housed all fish by population, in groups of 15–25, in 38-litre aquaria with aquatic plants provided for shelter. Females were kept isolated from males for at least 30 days before testing to standardize sexual responsiveness (Morris et al. 1996). Since this is the period of gestation, females used in experiments would not have mated successfully since last giving birth. Each test subject was allowed to acclimate for at least 10 min before all experimental trials. We performed all experiments in a windowless laboratory to control lighting conditions. Laboratory room temperature remained at  $27 \pm 2^\circ\text{C}$ .

## Experiments 1 and 2: Male Behaviour

We conducted two experiments to test for behavioural differences in pairs of size-matched males. The first experiment examined differences in male mating behaviour in direct male–female interactions and the second tested differences in competitive behaviour in direct male–male interactions.

### Set-up and procedure

We combined the two direct interaction experiments, male–female and male–male, for each trial and recorded the interactions with a Hitachi VM-H100LA Hi-8 camera. The fish were tested in an aquarium (30 × 15 cm) filled to 13.5 cm. A xenon lamp (Pelican Aqua KingLite 8D 4000 series submersible), which emits light across the visible and ultraviolet range, was placed above the aquarium.

For each trial we chose a female from a pool of previously untested females and acclimated her in the

experimental tank for at least 10 min. Males were from Nacimiento Huichihuayán or la Y-Griega Vieja populations. We acclimated a pair of males from the same population, one blue and one gold, for 10 min in individual visually isolated 1-litre containers. Males were matched for body size (standard length, SL) within 2 mm. After acclimation, a male, blue or gold, was placed in the test tank with the female and the interaction recorded for 10 min. We then removed the gold male and repeated the procedure with the other male morph. The initial male morph placed in the tank was systematically varied to balance the experiment. The female was then removed from the tank and the initial male reintroduced for the male–male interaction. We recorded this interaction for 10 min. We used 10 male pairs and each fish was used for only one set of trials.

### Analysis

We analysed the recorded interactions to quantify male reproductive and competitive behaviour patterns. We scored the incidence of two behaviours, chase and parallel approach, to assess differences in male behaviour towards females. For male–male interactions, we assayed chase, retreat and attack behaviours. Behaviours are fully described in Ryan & Causey (1989). The chase behaviour is characterized by a male chasing a retreating fish. A male performs a parallel approach when he swims closely parallel to the female but does not attempt to copulate. We did not observe transverse approaches or attempted copulations (Ryan & Causey 1989).

A retreating fish swims away from an approaching male, and attack behaviour occurs when a male makes contact with another fish with head or mouth, actively biting the fish (Ryan & Causey 1989). In male–male interactions, one male typically retreated quickly during an agonistic encounter. Agonistic interactions never escalated into physical contact and were at a level no greater than that expected from baseline behaviour of males housed for long periods in common aquaria. Depending on the behaviour, we analysed tapes at normal speed, slow speed, or frame-by-frame. We used a Mann–Whitney *U* test to evaluate differences in male behaviour by population. Since there was no significant effect of population on male behaviour, we pooled results from both male populations for analysis. We recorded the frequency of each behaviour for gold and blue males, and used a chi-square test on the null hypothesis of no difference between gold male attacks on blue males and vice versa. For the male–female interactions, we used a Mann–Whitney *U* test to compare the frequency of

parallel approaches and chase behaviour for gold and blue males.

### Experiments 3–5: Female Preference for Male Traits

In the remaining three experiments we examined female preferences for male body colour and behaviour. Experiment 3 tested female preference for male body colour and behaviour in live males, while controlling for male body size and shape. We used video animations in experiment 4 to standardize male body colour, size, shape and behaviour while testing female preference for colour. In experiment 5 we used colour filters that masked body colour in a pair of size-matched live males, to test female preference for behaviour. Male competitive behaviours occur over short distances (<10 cm) and males were separated by a minimum of 45.6 cm during choice tests.

#### *Experiment 3: set-up and procedure*

We tested females in a glass aquarium (76 × 31.5 cm and 30 cm deep) which had been filled to 17 cm. The tank was divided into three compartments, partitioned by dividers transparent in both the visible and ultraviolet range (Roehm Plexiglas GS2458) that had been attached to the tank with a continuous strip of silicone. The two end compartments were 15.2 cm long and the centre compartment was 45.6 cm long. The centre was further divided into three sections or 'zones' 15.2 cm long, which were marked on the glass with a wax pencil. We placed two xenon lamps above the two end compartments of the tank. In addition to the broad-spectrum xenon lamps, the room was illuminated with fluorescent ceiling lights.

For each trial, we chose two size-matched males (to ± 2 mm), one blue and one gold, from a pool of test fish. After a 10-min acclimation in individual, visually isolated 1-litre containers, one fish was placed in each side compartment of the test tank. A female test subject from the same population was placed in a Plexiglas cylinder in the centre of the tank for acclimation. She was then released from the cylinder and her position was continuously recorded for 10 min. The female was then returned to the cylinder. To control for side biases, we repeated the trial after switching the males between the end sections of the tank. We interpreted time spent in the 15.2-cm-wide zone nearest a given male, blue or gold, as a preference on the part of the female subject for that colour morph.

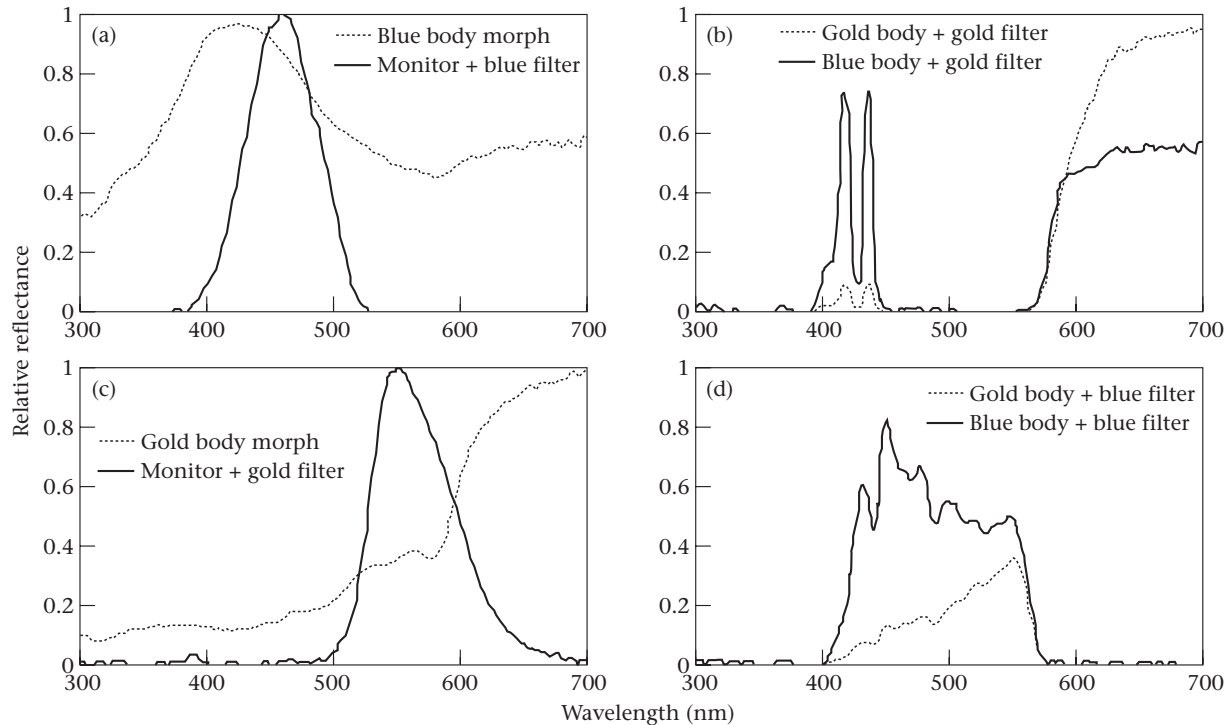
We systematically alternated the initial placement of the males (left or right). We used 60 females from the three *X. pygmaeus* populations, testing each female only once. Males were returned to the original pool and made available for retesting. We were unable to keep track of individual males, but males were continuously drawn from large outdoor stock populations (>100 adults), making it unlikely that individual males were tested more than twice.

#### *Experiment 4: set-up and procedure*

We used presentations of synthetic video animations to test female preferences for male body colour while

controlling male body size, shape and behaviour. We constructed a synthetic animation of a male *X. pygmaeus* (Rosenthal 2000a, b; Rosenthal et al. 2001b), with average characteristics based on field measurements of standard length and body depth from 177 males from all three populations of pygmy swordtails. We used 3D Studio Max 1.0 (Kinetix) on a Dell Optiplex GXPro computer for all modelling, animation and rendering, and a Targa 1000 board for digital-to-analogue and analogue-digital conversion of video signals. We derived fine-scale properties of the animated stimuli from morphometric measurements taken from preserved specimens in the Texas Natural History Collection, Texas Memorial Museum. We used callipers to measure standard length and nine measures of body thickness. The position of 24 landmarks on each specimen was recorded with a dissecting microscope and a camera lucida. We obtained outlines of body shape by placing specimens on an illuminated slide table to produce a high-contrast silhouette ( $N=17$ ). Each specimen was videotaped for <1 s with a Hitachi VM-H100LA Hi-8 camera. A single frame per specimen was digitized and the edge-finding function in Adobe Photoshop (Adobe Systems) was applied to produce a binary trace outline. Traces were then aligned over one another so that the snout-caudal peduncle axis was horizontal and scaled to match horizontal size across traces. A greyscale image in which each pixel reflected the mean grey value across the corresponding pixels in each source image was produced with the UTHSCA ImageTool program (developed at the University of Texas Health Sciences Centre at San Antonio, Texas, and available from the Internet by anonymous FTP at maxrad6.uthscsa.edu). A single trace that passed through the centre of the outline of the mean image was used as the base shape of the model fish. The body depth and fins were constructed according to the mean measurements of the corresponding landmark positions on the image, producing a wire frame model. We used Adobe Photoshop to create greyscale body colour, opacity and texture maps from arbitrarily selected digitized slides of one blue and one gold male. The maps were then applied to the wire frame, completing the three-dimensional model.

The applied motion was based on analysis of a 26-min video recording of live fish in the field. We used the mean swim velocity, turn velocity and turn angle to animate the synthetic body model. The final animation consisted of a single looped display of a male making an angled forward approach in a straight line, turning and swimming in a straight line off the screen. The background against which the fish was displayed was alternated in successive approaches between two monochromatic backgrounds, one representing average RGB (red/green/blue) pixel values taken from substrate in field videotapes of *X. pygmaeus* and the other average pixel values taken from the background spacelight (i.e. with open water as background). The presentation sequences were played on Panasonic AG-1970 S-VHS videocassette players and presented on two Panasonic TR-930B 9-inch monochromatic monitors. Colour filters (15.5 × 8 cm), blue on one end and gold on the other, were placed over each display screen. The colour filters were customized to match



**Figure 1.** Normalized intensity functions for reflectance of male body colour and radiance of video stimuli for (a) blue and (b) gold males; (c) and (d) show the reflectance functions in (a) and (b) under filtered conditions.

closely the spectral transmission curves of a blue and gold male morph. The brightness level of the monitor was reduced and the contrast increased by equal amounts on each monitor to minimize background transmission so that the animated fish was visible against a black background. **Figure 1** compares spectral measurements of light emitted from the monitor/filter combinations and reflectance spectra of male body coloration.

We tested females in an aquarium ( $76.8 \times 31.8$  cm) filled to a height of 21 cm, the height of the video display. The aquarium was divided into three 25.6-cm sections (left, right and centre), with a small clump of Java moss, *Vesicularia dubyana*, provided for cover in the centre section. A Panasonic TR-930B 9-inch monochromatic monitor abutted either end of the aquarium, facing forwards to reduce reflection from the filters on the aquarium glass. To reduce further the effects of glare from the filters, a 25-W incandescent soft white lamp was placed 90 cm above the centre of the tank and all other lights in the room were kept off.

We presented each female with 10 min of the monochromatic background on both sides followed by simultaneous presentation of the 300-s test stimuli. The subjects were then presented with a 10-min blank screen interval followed by the same set of stimuli presented on opposite sides of the set-up to control for potential side biases. The initial position of the monitors was systematically alternated to control for order effects. The time that the female spent in each section for the duration of each stimulus period was then recorded. Half of the trials began with the 'gold' monitor on one side of the tank and

the other half of the trials began with the 'gold' monitor on the other side.

#### *Experiment 5: set-up and procedure*

The experiment was set up in the same manner as experiment 3, except that identical colour filters were placed over the xenon lamps to mask male body colour. A 25-W red incandescent lamp was suspended 40 cm above the tank and provided the only additional light source. This provided just enough illumination by which to follow the female's movements. We lined the exterior of the male compartments with black felt to provide a black background. **Figure 1c, d** shows the reflectance spectra of males under filtered conditions.

For each trial, we chose two size-matched males (to  $\pm 2$  mm), one blue and one gold, from a pool of test fish and, after acclimation, placed one in each side compartment of the test tank. A female test subject from the same population was placed in a Plexiglas cylinder in the centre of the tank for acclimation. She was then released from the cylinder and her position was continuously recorded for 10 min. The female was then returned to the cylinder and the side each male was on was switched, to control for any side biases, and the procedure was repeated.

We systematically altered the colour of the filters used (blue or gold) so that half of the females were tested with blue filters and half with gold filters. The initial placement of each male (left or right) was also systematically altered. We interpreted time spent in the zone (15.2-cm section) nearest a given male, blue or gold, as a preference on the part of the female for that colour morph. After

each trial, the female test fish was transferred to a 'done' tank to avoid retesting subjects and the males were returned to the original pool and made available for retesting.

## Analysis

We performed a two-way repeated measures ANOVA (blue versus gold  $\times$  experiment  $\times$  population) on  $\ln(x+1)$ -transformed female response data to test for an overall colour preference and for response differences between experiments and populations. We subsequently analysed female preference test data in individual experiments by using a Wilcoxon signed-ranks test to compare female association times with gold versus blue stimuli. In experiment 5, the female preference test using colour filters, we also used a Wilcoxon signed-ranks test to analyse independently female responses under each filter (blue or gold). Net responses under each filter condition were then compared with a Mann–Whitney  $U$  test. In addition, for each female preference experiment we used a Kruskal–Wallis analysis of variance to compare responses across populations. Females that spent more than 90% of their time on one side of the test tank were excluded from all analyses. We used two-tailed statistical tests.

## RESULTS

### Experiment 1: Female–Male Interaction

This experiment tested colour-related differences in male mating behaviour by analysing direct interactions between individual males and females. The chase behaviour of males was not significantly different between the Nacimiento Huichihuayán and la Y-griega Vieja populations (blue males:  $U=6.5$ ,  $N_1=N_2=5$ ,  $P=0.207$ ; gold males:  $U=7.5$ ,  $N_1=N_2=5$ ,  $P=0.293$ ). Gold males chased females significantly more than blue males ( $\bar{X} \pm SE$ ; gold males:  $15.2 \pm 1.8$  chases; blue males:  $6.3 \pm 1.5$  chases;  $U=7.5$ ,  $N_1=N_2=10$ ,  $P<0.01$ ).

There was no significant difference in the frequency of parallel approaches by population (blue males:  $U=15.0$ ,  $N_1=N_2=5$ ,  $P=0.588$ ; gold males:  $U=10.0$ ,  $N_1=N_2=5$ ,  $P=0.598$ ). Gold and blue males did not differ significantly in the frequency of parallel approaches ( $\bar{X} \pm SE$ ; gold males:  $4.0 \pm 0.8$  parallel approaches; blue males:  $2.2 \pm 0.8$  parallel approaches;  $U=30.5$ ,  $N_1=N_2=10$ ,  $P=0.13$ ), although the trend favoured gold males.

### Experiment 2: Male–Male Interaction

This experiment analysed direct interactions between a pair of size-matched males, one blue and one gold. Gold males were significantly more likely to chase blue males than vice versa (chi-square test:  $\chi^2_1=10.9$ ,  $N=106$ ,  $P<0.001$ ). Gold males chased blue males a mean  $\pm SE$  of  $7.8 \pm 1.1$  times per interaction while blue males chased gold males  $3.6 \pm 1.2$  times. There was no significant difference in male behaviour between the Nacimiento Huichihuayán and la Y-griega Vieja populations for both

the frequency of chases (blue males:  $U=15.5$ ,  $N_1=N_2=5$ ,  $P=0.525$ ; gold males:  $U=17.0$ ,  $N_1=N_2=5$ ,  $P=0.341$ ) and retreats (blue males:  $U=10.0$ ,  $N_1=N_2=5$ ,  $P=0.598$ ; gold males:  $U=12.5$ ,  $N_1=N_2=5$ ,  $P=1.00$ ).

### Experiments 3–5: Female Preferences for Male Traits

We first combined data from the three mate choice experiments to examine whether females from each population were behaving differently across and within experiments. Females overall showed a significant preference for blue when response data from all three experiments were combined (ANOVA:  $F_{1,1}=6.860$ ,  $P=0.01$ ). The preference for blue varied significantly across populations ( $F_{2,2}=3.181$ ,  $P<0.05$ ). Neither experiment (ANOVA:  $F_{2,3}=0.678$ ,  $P=0.57$ ) nor the interaction of population by experiment (ANOVA:  $F_{4,6}=0.418$ ,  $P=0.87$ ) had a significant effect on preference.

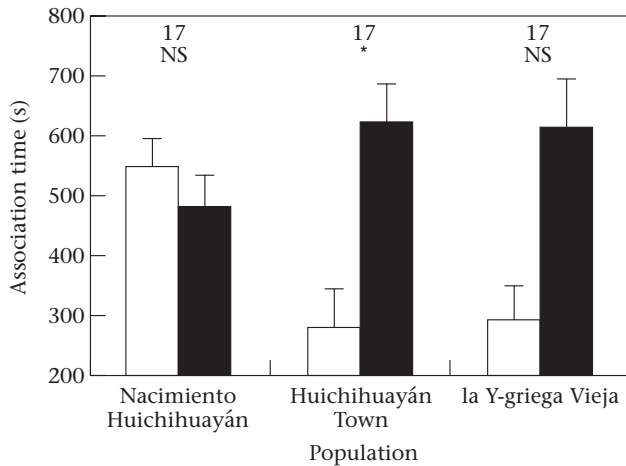
We performed a post hoc Fisher's least significant difference test (LSD) to examine pairwise differences in response between populations. La Y-griega Vieja and Huichihuayán Town were not significantly different from one another ( $P=0.856$ ) while Nacimiento Huichihuayán females showed a significantly greater response to gold than both la Y-griega Vieja ( $P=0.01$ ) and Huichihuayán Town ( $P<0.01$ ) populations.

### Experiment 3: Live Blue and Gold Males

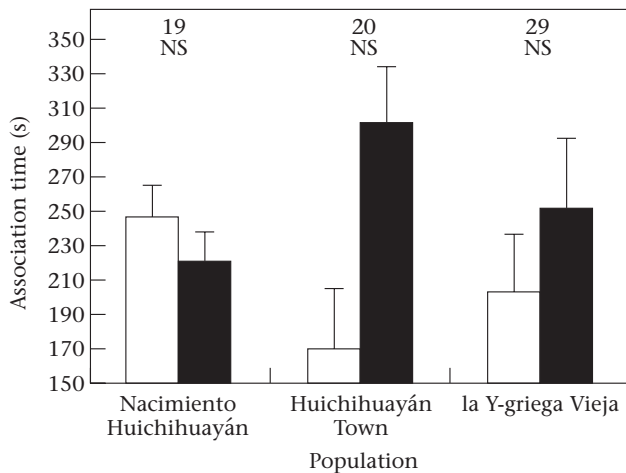
In the third experiment we presented females with live males to test female preference for the full male phenotype. Females showed no significant preference for male colour (Wilcoxon signed-ranks test:  $Z=1.619$ ,  $N=51$ ,  $P=0.11$ ) when data for all populations were combined. There was a marginally nonsignificant trend suggesting differences in preference between populations (Kruskal–Wallis test:  $H_2=5.479$ ,  $P=0.06$ ). When populations were considered separately, Huichihuayán Town females showed a significant preference for blue males ( $Z=2.291$ ,  $N=17$ ,  $P<0.05$ ). Nacimiento Huichihuayán ( $Z=-0.517$ ,  $N=17$ ,  $P=0.60$ ) and la Y-griega Vieja ( $Z=1.412$ ,  $N=17$ ,  $P=0.16$ ) females showed no significant preference (Fig. 2).

### Experiment 4: Video Stimuli

In this experiment we used video presentations to test female preference for colour while male size and behaviour were held constant. The results failed to reject the null hypothesis of no female preference for blue versus gold (Fig. 3). This result holds when data sets for the three populations were combined (Wilcoxon signed-ranks test:  $Z=1.219$ ,  $N=68$ ,  $P=0.22$ ) and when the data sets were analysed individually by population (Nacimiento Huichihuayán:  $Z=-0.284$ ,  $N=19$ ,  $P=0.78$ ; Huichihuayán Town:  $Z=1.503$ ,  $N=20$ ,  $P=0.13$ ; la Y-griega Vieja:  $Z=1.004$ ,  $N=29$ ,  $P=0.32$ ). However, the overall results paralleled those of the female choice experiments with live males (Kruskal–Wallis test:  $H_2=0.281$ ,  $P=0.87$ ).



**Figure 2.** Mean association time (s) of *X. pygmaeus* females with live gold (□) and blue (■) males (experiment 3). Sample sizes are given above each comparison. \* $P < 0.05$ .

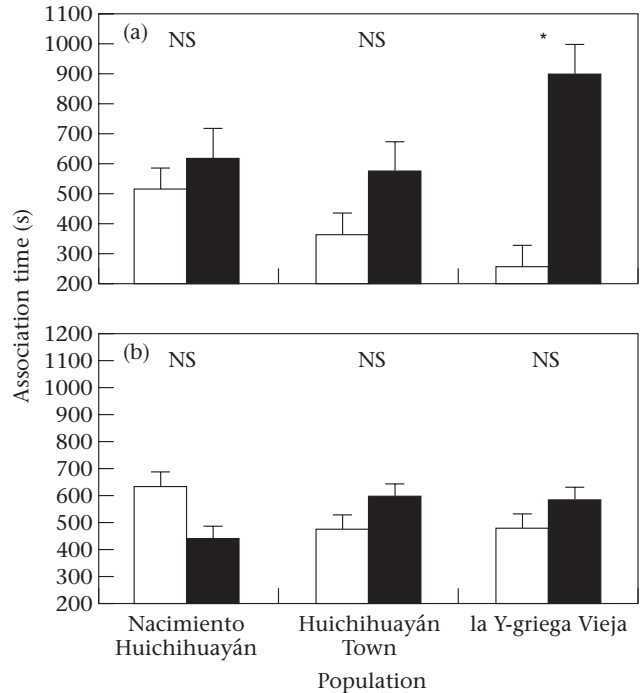


**Figure 3.** Mean association time (s) of *X. pygmaeus* females with synthetic animations of gold (□) and blue (■) males (experiment 4). Sample sizes are given above each comparison. \* $P < 0.05$ .

Populations did not differ significantly in their preferences ( $H_2 = 1.53$ ,  $P = 0.46$ ) within the experiment.

### Experiment 5: Live Males with Masked Body Colour

In this experiment, we attempted to mask male body colour to test female preference for male mating behaviour associated with each colour morph. There were significant preferences for blue males under gold filters in la Y-griega Vieja population (Wilcoxon signed-ranks test:  $T = 1.0$ ,  $N = 10$ ,  $P < 0.01$ ) and when the data for all populations were combined ( $Z = 2.643$ ,  $N = 30$ ,  $P < 0.01$ ; Fig. 4a). Nacimiento Huichihuayán ( $T = 23.0$ ,  $N = 10$ ,  $P = 0.646$ ) and Huichihuayán Town ( $T = 14.0$ ,  $N = 10$ ,  $P = 0.17$ ) females showed no significant preference (Fig. 4a). Populations did not differ significantly in their preferences (Kruskal–Wallis test:  $H_2 = 3.41$ ,  $P = 0.18$ ).



**Figure 4.** Mean association time (s) of *X. pygmaeus* females with gold (□) and blue (■) males (experiment 4) under (a) gold and (b) blue filters. Sample sizes are given above each comparison. \* $P < 0.05$ .

Females did not show any significant preferences when males were presented under a blue filter (Wilcoxon signed-ranks test: all  $Z = -0.051$ ,  $N = 30$ ,  $P = 0.96$ ; Huichihuayán Town:  $T = 20.0$ ,  $N = 10$ ,  $P = 0.44$ ; Nacimiento Huichihuayán:  $T = 17.0$ ,  $N = 10$ ,  $P = 0.28$ ; la Y-griega Vieja:  $T = 24.0$ ,  $N = 10$ ,  $P = 0.72$ ; Fig. 4b). Populations did not differ significantly in their preferences (Kruskal–Wallis test:  $H_2 = 2.16$ ,  $P = 0.34$ ).

Female preference for blue males was significantly stronger under the gold filter than under the blue filter (Mann–Whitney  $U$  test:  $Z = 2.06$ ,  $N_1 = N_2 = 30$ ,  $P = 0.04$ ).

## DISCUSSION

One mechanism for the maintenance of a stable polymorphism within a species is balancing selection. Our results suggest that in *X. pygmaeus*, female preference for male morphs is counterbalanced by male mating and agonistic behaviour. Direct interactions between males and females, and between males, showed that gold males are more aggressive towards both females and blue males. Gold males chased females significantly more than did blue males, suggesting that gold males may be more successful at forcing copulations. Gold males were more likely to prevail in agonistic interactions with blue males, which may confer an advantage in competition over mates or foraging sites.

When females were allowed to choose between morphs, preferences tended to favour blue males in two of three populations. In one of those populations, females allowed to choose between blue and gold males under full illumination (experiment 3) had a significant preference

for blue males. We observed similar trends for female responses to synthetic stimuli, which were identical in behaviour and morphology but differed in colour.

In the final experiment, when we masked the body colour of live males, females showed a significant preference for blue males under gold filters, when gold males appeared gold and blue males appeared black (Fig. 1). Females did not show a preference under the blue filters, when the gold colour was masked. The results suggest that a component of the female preference for blue males may be an aversion to gold males; when only gold can be readily detected, females prefer blue males.

Taken together, the results of the last three experiments suggest that both the colour and behaviour of gold males act in concert to structure female preference. Females showed a significant preference for blue males only when both the colour and behaviour of gold males were present. When filters eliminated gold colour, or video playbacks eliminated behavioural differences between gold and blue males, females showed no significant preference.

It is unlikely that male–male competition in the live male choice tests could have influenced female preference for male colour. In field observations and in the male–male interaction experiments of the present study, male competitive interactions occurred uniformly across distances of less than 10 cm. In the choice experiments, males were separated from each other by the 45.6-cm centre compartment. Furthermore, in experiment 5 we used and alternated colour filters to test female preference for behaviours associated with a colour phenotype while controlling for female colour preference. If male–male competition affected female choice we would expect significant results under both filter conditions in the last experiment. However, females showed a substantial difference in response under different colour filters, reinforcing the idea that both colour and behaviour structure female preference.

We found substantial differences in female preferences between populations. Females from the Huichihuayán Town and la Y-griega Vieja populations preferred blue males in the mate choice experiments whereas Nacimiento Huichihuayán females showed no preference in any trial.

The interpopulation variation in preference patterns for colour morph parallels that found for body size (Morris et al. 1996) and other male traits (Rosenthal 2000a). The lack of female preference for colour morphs at the Nacimiento Huichihuayán site, where densities of fish predators are the highest among all fish populations surveyed, may reflect a general loss of female mate choice behaviour in areas of high predation (Rosenthal 2000a; Rosenthal et al. 2001b).

The relation between body size preference and preference for blue males is particularly intriguing. In *X. pygmaeus*, the blue/gold polymorphism is independent of body size and males are fixed at the Y-linked *P* locus for the *s* ('small') allele. Some male *X. pygmaeus* are abnormally large, a phenotype that does not appear to have a heritable component (Dries et al. 2001). In the closely related *X. nigrensis* and *X. multilineatus*, the *con* allele

associated with gold colour is restricted to males with the *s* allele (Kallman 1989). Males of these species with alleles conferring large body size are blue. In *X. nigrensis* and *X. multilineatus*, therefore, coloration is strongly correlated with body size. The lack of preference for blue males at the Nacimiento in this study, and the loss of preference for large size at the Nacimiento in Morris et al.'s (1996) study, involve traits that are closely associated in related species.

A number of analyses have failed to discern any fitness cost to female *X. pygmaeus* of mating with abnormally large males (Rosenthal 2000a) or any heritable component to large size (Dries et al. 2001). The apparent loss of preference for body size in Morris et al.'s (1996) study may not, as initially suggested, arise from selection against mating with these large males per se, but from a loss of female preference in general, owing to high predation risk (Rosenthal et al. 2001b). Frequencies of blue males are lowest at the Nacimiento Huichihuayán, which may reflect the lack of a mate choice advantage for blue males there. Gold males may be prevented from spreading to fixation in the population by migration or by countervailing selection against the gold phenotype, such as a higher level of predation risk.

Our results suggest that the colour polymorphism in *X. pygmaeus* may in part reflect a balance between female preference for blue males and increased aggression in gold males. The marked differences in reflectance spectra between blue and gold males, and the pronounced behavioural differences between the two morphs, make it likely that additional factors are important for maintaining the polymorphism. Swordtails' major predators, Mexican tetras, *Astyanax mexicanus*, show a visual preference for larger and more ornamented males (Rosenthal et al. 2001a), so they may be able to discriminate between morphs. Heterogeneity in the background and transmission characteristics of the visual environment may also help to maintain the polymorphism. Finally, there may be a genetic correlation between colour morph and other traits under selection; one compelling possibility is that there may be an immune system cost to increased aggression (Zuk 1996) or carotenoid pigment expression (Hill 1999) in gold males.

The discrete blue/gold variation in male pygmy swordtails is one of the more striking single-locus polymorphisms found in vertebrates. These visually divergent phenotypes are straightforward to score, and many of the factors influencing their fitnesses can be readily measured. The colour polymorphism in *X. pygmaeus* and its close relatives may provide a powerful model system for studying the evolution of sexual traits.

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