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Mate choice for a novel male phenotype in zebrafish, *Danio rerio*

M. Aaron Owen [1], Karl Rohrer, Richard D. Howard*

Department of Biological Sciences, Purdue University, West Lafayette, U.S.A.

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Studies of mating preferences contribute to understanding the evolution of male secondary sexual traits. How females respond to novel male characteristics may lend additional insight into their mating preferences and subsequent mate choice patterns. Studies on several species have shown that females prefer males with manipulated or novel phenotypes. However, few studies have investigated the mechanisms that underlie female preferences. Adopting a relatively underutilized technology in studies of mate choice (genetic modification), we used transgenic zebrafish (*Danio rerio*) to evaluate female preference for normal versus novel transgenic (red GloFish™) males. We conducted four mate choice experiments in which females differed in rearing history to determine whether female mating preferences are influenced by population history, sexual imprinting or colour of food in their diet. We used a two-male association protocol in all four experiments as well as a one-male mating protocol in one experiment. In all four experiments, the association protocol demonstrated that females preferred novel transgenic males to normal males. The one-male protocol revealed no pattern of female mating preference; instead, mating patterns were strongly influenced by male coercion. Overall, our results suggest that zebrafish may possess a sensory bias for the colour red; however, more research using both closely related species and other colour morphs of GloFish™ are needed to resolve this issue. Our study exemplifies the utility of using transgenic organisms to study mate choice, and we propose that future studies incorporate this untapped technology.

Darwin (1859, 1871) proposed the concept of sexual selection in which some individuals achieve higher mating success because of advantages in mate choice and mate competition. Here we concentrate on how male mating success might be influenced by female mate choice in our study organism, *Danio rerio*; elsewhere, we address the role of mate competition on mating success in this species (R. D. Howard & K. Rohrer, unpublished data). In many species, females choose to mate with males that possess particular secondary sexual trait expressions, and the degree of elaboration of these traits in subsequent generations may be determined by the overall pattern of female mate choice in a population (Andersson 1994; Howard & Young 1998; Widemo & Saether 1999).

Female preferences for male traits are often assumed to be genetically based (Andersson 1994; Hörster et al. 2000) and may have evolved because discriminating females obtain some reproductive advantage as a consequence of their choice either in terms of direct benefits (e.g. access to high-quality resources or enhanced fertilization success) or indirect benefits (e.g. production of genetically superior offspring; Trivers 1972; Kirkpatrick & Ryan 1991). Many factors may influence mate preferences, such as ontogenetic learning (especially sexual imprinting: Lorenz 1935 cited in Bateson 1978; Immelmann 1975; Witte et al. 2000), mate choice copying (Dugatkin 1992; Galef et al. 2008; Godin & Hair 2009), relative frequency of male morphs (Ehrman 1966) and sensory biases (Endler & Basolo 1998). Any of these factors could modify mate choice patterns and thus affect the evolution of male secondary sexual characteristics (Rosenqvist & Houde 1997).

It is uncertain how male secondary sexual traits arise in a population before they become the object of female mate choice. Evolutionarily, such novel traits should ultimately result from genetic mutations but could also be initiated by environmental induction (West-Eberhard 2003). Determining how females respond to novel male characteristics may lend insight into the evolution of mating preferences and subsequent mate choice patterns. Females may benefit from preferences for novel (or altered) male characteristics if such pairings provide a reproductive advantage such as increased offspring genetic heterozygosity or avoidance of inbreeding depression (Kokko et al. 2007). Alternatively, females that preferentially mate with males with novel characters might obtain no reproductive advantage or even a disadvantage. In the latter situation, selection is expected to

* Correspondence: R. D. Howard, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, U.S.A.
E-mail address: rhoward@purdue.edu (R. D. Howard).
[1] M. A. Owen is now at the Department of Biology, Queens College, Flushing, NY 11367, U.S.A., and The Graduate Center, The City University of New York, New York, NY 10016, U.S.A. *E-mail address:* mowen@gc.cuny.edu (M. A. Owen).

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favour females that reject males with novel phenotypes. However, previous studies have shown that females may prefer males with novel traits when given a choice between manipulated and normal male phenotypes (e.g. Burley et al. 1982; Schlupp et al. 1999; Witte & Curio 1999).

Production of novel male traits using genetic modification is an untapped technology that could prove useful in determining how females respond to novel male phenotypes. In this study, we investigated female mating preferences for a novel male trait in zebrafish, *Danio rerio*, by providing females a choice between normal (i.e. wild-type) males and novel red transgenic GloFish™ males whose coloration is almost entirely red (and who are hereafter referred to as 'red' males). Zebrafish are a model organism for many fields of biological research including development, neurophysiology and genetics; however, relatively few studies have investigated their mating and sexual behaviour (e.g. female mating preferences: Pyron 2003; Spence & Smith 2005; Snekser et al. 2006; courtship behaviour: Darrow & Harris 2004; male–male competition: Spence & Smith 2006). Spence et al. (2006a) concluded that the opportunity for sexual selection is weak in zebrafish based on an analysis of offspring genotypes produced by sires and dams; however, other studies provide behavioural evidence for mate choice (Pyron 2003; Hutter et al. 2010).

We conducted four mate preference experiments (Table 1) in which females differing in rearing history were given the opportunity to select between the two types of males. In experiment 1, the rearing conditions and social environments of females were not manipulated. Tested females came from populations in which both colour morphs had been well represented in both sexes for three generations. The goal of this experiment was to determine whether females in our source populations possessed a mating preference for or against the novel red male morph. This experiment was necessary given the conflicting views of the likelihood of mate choice in this species, and in particular, the lack of female mate preference for red transgenic males that was demonstrated in a previous study (Snekser et al. 2006).

In experiment 2, the females tested came from three populations, all of which began with a 75% frequency of the red morph. After six generations, however, one of the populations contained an equal mix of red and normal morphs while the other two populations had become nearly fixed for either the red morph or the normal morph. The goal of this experiment was to determine whether the continuous decline of one morph in a population across generations was caused by a divergence in female mating preferences for red or normal males.

In experiment 3, we tested females that were reared from the fry stage in groups differing in the ratio of red to normal individuals. The goal of this experiment was to determine whether early social experience (sexual imprinting) influences subsequent mate preference. Sexual imprinting can be defined as a process by which young individuals learn general, species-specific characteristics, usually from parents or siblings, which are then used as cues for species recognition and mate preferences (Irwin & Price 1999; Freeberg 2000). Sexual imprinting has been most extensively studied in birds (reviewed in ten Cate & Vos 1999), but has also been investigated in some mammals (Kendrick et al. 1998; Penn & Potts 1998), fish (Breden et al. 1995; Verzijden & ten Cate 2007) and invertebrates (e.g. Rutledge et al. 2010). The evolutionary significance of sexual imprinting could be two-fold (Freeberg 2000): it may allow individuals to form stable responses to 'biologically relevant objects' and may result in reproductive isolation if imprinting influences behavioural processes such as courtship. The early social environment of zebrafish has been shown to influence behaviours such as shoaling, with fish preferring to shoal with phenotypes similar to those with which they were raised, regardless of their own phenotype (Engeszer et al. 2004; Moretz et al. 2007; Spence & Smith 2007). It is not known, however, whether a similar learning mechanism could also shape female mating preferences.

In experiment 4, we tested females that were reared on an all-green diet in contrast to the fish used in the previous three experiments that had been reared on red food. The goal of this experiment was to determine whether female mating biases could be influenced by behaviours unrelated to mating (Kirkpatrick 1987). Previous experiments on food colour preferences in zebrafish as well as in other small fish species have revealed that individuals prefer red objects when foraging (Ibrahim & Huntingford 1989; Rodd et al. 2002; Spence & Smith 2008). It is not known, however, whether a preference for red food might influence a preference for red males as mates.

Thus, we tested the following three hypotheses. (1) If females have a mate preference, then they should associate more, mate quicker or more often with either a normal male or a red male. In particular, if a novel male phenotype influences preference, then females should prefer red males. (2) If sexual imprinting influences mate preference, then females should prefer to mate with the male phenotype with which they were reared prior to sexual maturity. Specifically, if females were reared solely with normal males, then they should prefer normal males over red males as mates; the converse is predicted if females are reared solely with red males. (3) If coloration of diet influences subsequent mate preference, then females should prefer males more similar in colour to their diet. That is, females reared on a red food diet should prefer red males; females reared on a green food diet should not show a preference for red males.

We also tested two assumptions of our predictions. In our experiments, we assumed that any association preference observed indicates a mating preference rather than some other type of preference (e.g. a shoaling preference). Because most mating activity in this species occurs immediately after 'lights on' in the

Table 1
Summary of experiments and their goals

Experiment	Group designation	Description	Question	Type of protocol used
1	Control	Adults from six populations differing in frequency of red morphs	Is there a general preference for or against the red male morph?	Two-male association tests
2	Differing population history	Adults from three populations (red morph frequency equalled 8%, 50%, 88%)	Do prior population trajectories influence preference patterns?	Two-male association tests
3	Ontogenetic effects: sexual imprinting	Offspring of >50 parental groups reared in one of four groups (all red; all normal; 9 red:3 normal; 3 red:9 normal)	Do social associations prior to sexual maturity influence mate preference patterns?	Two-male association tests followed by one-male mating latency tests
4	Ontogenetic effects: diet colour	Offspring of adults from several populations reared entirely on a green diet	Does colour of diet influence subsequent mate preference patterns?	Two-male association tests

morning (simulating dawn) (Eaton & Farley 1974; Spence et al. 2006b; Lawrence 2007), we also tested female preference in the afternoon, when any observed association would be more likely to reflect a nonmating preference (e.g. a shoaling preference). In addition, we provided females a choice between a red and normal female in the morning. Secondly, because we assumed that any preference for red transgenic males was influenced by their red coloration rather than their UV reflectance, we provided females a choice between a red male and a green transgenic GloFish™ male.

In all experiments, we used a two-male association protocol in which female preference was inferred by the relative amount of time she spent in proximity to each male. We also used a one-male mating protocol in one experiment (experiment 3, Table 1) in which female preference was inferred by the female's latency to mate (Wagner 1998). Both of these testing protocols have been used to assess mate preference in a variety of species (Howard et al. 1998; Pyron 2003; Sisodia & Singh 2004; McGuigan et al. 2008); however, there is some disagreement about which protocol is better (Wagner 1998).

METHODS

Study Organism

Zebrafish, *Danio rerio*, are native to southern Asian countries including India, Bangladesh, Nepal, Myanmar and Pakistan (Laale 1977; Engeszer et al. 2007). They inhabit stagnant or slow-moving freshwater ponds and rice ponds (Spence et al. 2006b). Normally, zebrafish are light brown with lateral blue and either gold (males) or silver (females) alternating stripes. Red GloFish™ are derived from a strain of zebrafish that is transgenic for cDNA encoding for a red fluorescent protein (RFP) cloned from the IndoPacific sea anemone (*Discosoma* sp.). The RFP transgene produces a red colour that can be seen in normal light but which is enhanced under ultraviolet light (UV); the red phenotype is expressed similarly in hemizygous and homozygous individuals of both sexes. Zebrafish possess four cones in their retina with absorbance peaks in the UV (362 nm) (Robinson et al. 1993), blue (415 nm), green (480 nm) and red (570 nm) (Hughes et al. 1998).

Females can mate daily, producing up to several hundred eggs each day (Spence et al. 2008). Ovulation is induced by male gonadal pheromones (Spence & Smith 2006; Spence et al. 2008), and spawning is triggered by light at dawn (Eaton & Farley 1974). Breeding activities can be regulated in the laboratory using an automated photoperiod such that mating activities begin at 'lights on'.

Rearing

Zebrafish used in our experiments were initially obtained from a commercial supplier (Yorktown Technologies L. P., Austin, TX, U.S.A.). Red and normal individuals came from 19 source populations which RDH established in 2007; each population was formed by stocking each of 19 640-litre tubs with 50 fry that were hemizygyous for the RFP gene. Hemizygous individuals were used to ensure that the initial frequency of the RFP gene in each population was 50% (R. Howard, W. Muir & A. Ragavendran, unpublished data). The populations were created for an ongoing, long-term investigation designed to test the 'Trojan Gene effect' predicted from a computer model (Muir & Howard 1999). The green GloFish™ males were also obtained from Yorktown Technologies in May 2011 and immediately used in testing.

Because of the timeline of our four mate preference experiments involving red males and normal males, different generations of the source populations were used for each experiment. In some experiments, we tested the adults collected; in other experiments, we used offspring of collected adults. All fish collected were housed in 39-litre aquaria and maintained in a 15:9 h light:dark room with a recirculating water system maintained at 27 °C. All groups, except the green-diet group, were reared on an all-red food diet: AP200 when fry, live Artemia and Brine Shrimp Flake Food (Aquatic Ecosystems, Inc., Apopka, FL, U.S.A.) when juveniles or adults.

Experiment 1: Source Population Mating Preferences

Fish used in this experiment had been reared for three to four generations in the source populations. We collected adults from six source populations in which the frequency of the red morph ranged from 50% to 70% at the time of collection. The adults collected were used directly in association tests.

Experiment 2: Effects of Different Population History

Fish used in this experiment were collected from three source populations after they had been reared for six generations. As expected from Hardy–Weinberg predictions, the first generation produced by the hemizygous red parents contained 75% red individuals. Across the six generations, the three populations used in this experiment had diverged in morph frequency: one population had increased from 75% red morphs in the F_1 generation to 88% red morphs (referred to below as the 'red-morph population'); the second population had declined from 75% red morphs to 8% red morphs (referred to below as the 'normal-morph population'); the third population had declined from 75% red morphs to 50% red morphs (referred to below as the 'equal-morph population'). The adults collected were directly used in association tests.

Experiment 3: Ontogenetic Effects: Sexual Imprinting

Fish used in this experiment were offspring produced from sixth-generation adults collected from several of the source populations. The percentage of red individuals in the source populations used in this experiment varied from 25% to 60%. To control for the possibility of inheriting a bias from any one population, more than 50 parental groups ranging from one male:one female groups to up to three male:three female groups were bred in all combinations (red female–red male, normal female–normal male, normal female–red male, red female–normal male). Parental groups never contained individuals from the same source population. If a group of parental fish did not produce young for a few consecutive days, they were replaced. Each parental group spawned in one of six 39-litre aquaria with an 1150 cm^3 plastic container with a mesh lid and green plastic plants projecting through the mesh (referred to as a 'breeding basket' hereafter).

Each day, breeding baskets were removed from parental aquaria and held for 7 days to allow young to develop. Fry hatch 2–3 days after spawning. Colour phenotype of fry was determined when 21 days old using a DR46B Dark Reader Transilluminator. Fry were placed in a small petri dish, collected using small, plastic pipettes, and placed into four treatment groups (Fig. 1): 12 normal (12N), 12 red (12R), nine red and three normal (9R/3N), and three red and nine normal (3R/9N). Sample sizes varied because of differential mortality of test fish: 19 replicates of both the 9R/3N and 3R/9N groups, 10 replicates of the 12N groups, and five replicates of the 12R groups.

Fry from each replicate of each treatment group remained in their own 10-litre aquaria until they were approximately 5 mm in length (about 6–8 weeks of age), after which each replicate was transferred to its own 39-litre aquaria. Maturity was confirmed by the presence of fertilized eggs found in breeding baskets in each aquarium after individuals in each replicate were about 12 weeks old.

Time to maturity ranged from 12 to 16 weeks; however, not all fish survived to maturity. At time of collection, there was an average

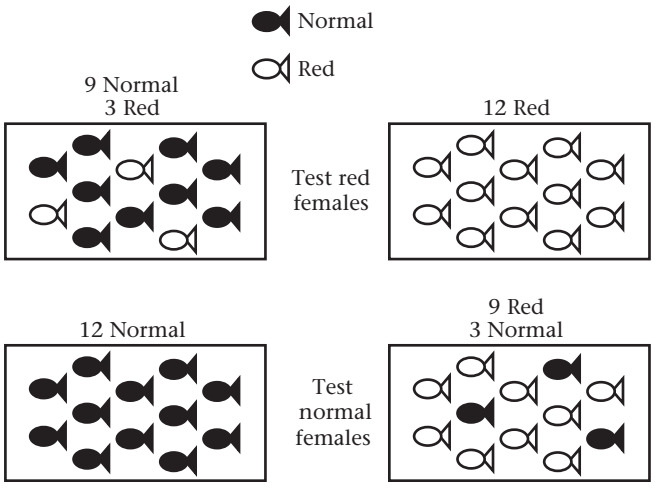


Figure 1. The four treatment sets used to determine whether early social experience influenced subsequent mate preferences in zebrafish. All sets contained 12 fish, but ratios of red to normal males differed.

of 10.7 fish per replicate (range 7–12 fish). The sex ratio of all replicates was male biased, averaging 6.3 males and 4.4 females per replicate. Mean ± SD size (standard body length) of males and females was 2.79 ± 0.26 cm and 2.78 ± 0.23 cm, respectively, and did not differ at time of maturity (t test: $t_{567} = 0.21$, $P > 0.83$). Gender was determined by the presence (female) or absence (male) of the external cloaca found just anterior to the ventral fin.

Experiment 4: Ontogenetic Effects: Diet Colour

In this experiment, our goal was to determine whether females show transference of food colour experienced during rearing to their subsequent association preferences of males. Fish used in this experiment were offspring produced from eighth-generation adults collected from several of the source populations that still contained appreciable frequencies of both colour morphs. Similar to experiment 3, crosses were conducted by pairing males and females of both morphs from different source populations to control for an inherited bias that might exist in any one population. Breeding and rearing protocols were similar to those described in experiment 3, except offspring were not segregated by colour morph status, and all offspring were reared on green AP100 fish food when fry, then switched to green Omega-one veggie flake food (Omega Sea Ltd, Sitka, AK, U.S.A.) when juveniles and adults. Once female offspring of both morphs attained sexual maturity, we tested them for their association preference for males of both colour morphs.

Testing Protocols

Two-male association test protocol

In our association tests, we assumed that the relative percentage of time that a female spends near each of two physically separated males (normal or red in our case) is directly related to her relative mating preference for the males. Our use of three aquaria in these tests eliminated the influence of olfactory cues, as we were explicitly testing for association preferences based on a visual cue (i.e. morph coloration). Olfaction is known to stimulate ovulation in females (van den Hurk & Lambert 1983; Lawrence 2007) and to be involved in kin recognition to reduce chances of mating with close kin (Gerlach & Lysiak 2006); however, both visual and olfactory cues are used in sex recognition (Hutter et al. 2010).

We tested each female only once; however, we selected males haphazardly for each trial and may have used them in multiple trials. Males and females used together in the same trial were never taken from the same population. In addition, males used in the same trial were never obtained from the same aquarium to ensure that no prior dominance relationship existed. In each trial, the two males used were within 1 mm in standard body length of each other to control for effects of differential male size on female preference.

We used an association testing protocol because previous results showed that aggressive male–male interactions can influence female choice in zebrafish when males and females have physical access to each other (Spence & Smith 2005). Under these conditions, females may mate with the more dominant male, but this choice may not necessarily reflect the female's preference; rather, it could result from male coercion. Thus, association tests eliminated complications of male–male interactions.

Two-male trials took place in three 30 × 20 × 15 cm (10 litre) aquaria aligned end to end (Fig. 2). The central aquarium contained the female; each of the two outer aquaria contained one male. We covered all aquaria on all sides except the side(s) facing an adjoining aquarium. We observed male and female activity from a mirror tilted at approximately a 45° angle above the aquaria. We placed males differing in colour morph randomly in either end aquarium and used a white partition to keep them within the closest 10 cm area to the central aquarium containing the female. The central female aquarium was divided into two outer choice zones and one central neutral zone, each 10 cm in length. Test fish were transferred from their holding aquaria to the test aquaria the evening before the trial. The three aquaria were separated by black partitions at all times prior to testing.

A trial consisted of two 10 min association tests that began immediately after 'lights on'. Initially, the female was confined to the neutral zone using clear partitions. The black partitions separating the three aquaria were removed so that the female could view both males. Females were kept in the neutral zone for an acclimation period of 5 min, after which the clear partitions were removed allowing the female to move freely in her aquarium, and the first 10 min association test was conducted. After this first association test, the black partitions were replaced and the female was again confined in the neutral zone using the clear partitions. Male aquaria were then switched to opposite sides of the female aquarium and the second association test was performed to test for a possible side bias. The black partitions were removed, and, after another 5 min acclimation period, the clear partitions were removed and the second 10 min association test was conducted.

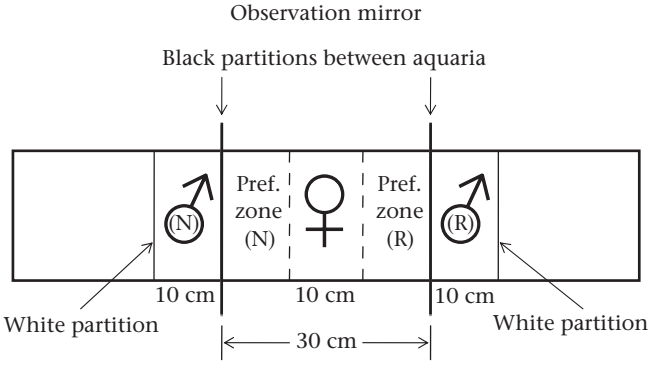


Figure 2. Testing arena. A red male (R) and a normal male (N) were randomly placed on either end of the female aquarium and forced to stay within the closest 10 cm to the central female aquarium using white partitions. Black partitions blocked fish from visual access to any other fish prior to testing.

After the entire trial, males were placed back in their original holding aquaria. Test aquaria were rinsed and filled with fresh water between trials to remove any residual pheromone cues.

Total choice time was defined as the total amount of time a female spent in both choice zones (time spent in the neutral zone was excluded from analyses). Preference time for each male was measured as the proportion of time a female spent in his choice zone divided by her total choice time. A male was designated as 'preferred' if the female spent at least 60% of her total choice time in his choice zone; females that did not show a preference of at least 60% for one male were excluded from analyses. We set this conservative criterion a priori to ensure that females were actually expressing a choice between the two males, and that the observed differences in choice times for each male (e.g. 51% versus 49%) were not due to chance. However, all results remained significant (i.e. $\alpha < 0.05$) when the 60% criterion was relaxed. Females that spent more than 80% of their total choice time in either the left or the right choice zone for both tests of a trial were considered to have a side bias and excluded from analyses.

In experiment 1, we tested 29 females of each colour phenotype but eliminated nine trials due to side biases and two additional trials because the females did not meet the 60% total choice time criterion; thus, 24 red females and 23 normal females were used in analyses. In experiment 2, we tested 52 females (21 red females and 31 normal females) but eliminated 12 trials due to side biases and nine trials that did not meet the 60% criterion. In experiment 3, we tested 104 females (26 red females from the 3R/9N and 12R treatment groups and 26 normal females from the 9R/3N and 12N treatment groups). No trials were eliminated due to side bias, but 46 trials were eliminated because females did not meet the 60% criterion. In experiment 4, we tested 36 females (16 red females and 20 normal females); no trials were eliminated due to side bias, but 13 trials were eliminated because females did not meet the 60% criterion.

One-male mating test protocol

In our one-male mating tests, we assumed that strength of female preference was directly related to how quickly she mated after encountering a male (i.e. a shorter latency time is assumed to indicate stronger preference) or to the number of mating bouts between the male and female. This testing protocol was only used in experiment 3. Each one-male mating test was conducted the day after the corresponding association test was performed using the female tested and one of the two males from the association test; that is, either the male with the higher association time ('preferred male') or the lower association time ('nonpreferred male'). Preferred males and nonpreferred males could be either red or normal in coloration.

Half of the females in each treatment group were assigned to a preferred male and the other half were assigned to a nonpreferred male. The initial female was randomly paired with one of the male types, after which the selected male to be paired with the female was alternated. Immediately after the association test trial, the assigned male was placed in the female's association test aquarium until late afternoon (the unassigned male was placed back in his original aquarium). The pair was then transferred to another 10-litre aquarium that contained a breeding basket. The fish were kept overnight in this aquarium whose ends were covered.

Fish behaviour was recorded using the ClearVision 4 DVR System (Inter-Pacific, Inc., Northbrook, IL, U.S.A.) for 1 h after 'lights on' the following day. Time to first mating and number of mating bouts were recorded for each pair. We considered a mating bout as occurring only if both the male and female showed quiver behaviour (Darrow & Harris 2004).

Of the 58 females that met the 60% criterion in the association trials in experiment 3, only 17 were observed to mate in the 1 h observation period of the one-male mating tests. Because latency to mating could either result from a female's preference or from male coercive behaviour in forcing females to mate, we tested for a relationship between mating latency and male chasing behaviour prior to the initial mating. Unfortunately, only 17 tests were available for this analysis because many data files were lost from computer memory when early files were written over with later files. In the 17 tests, six of the males were preferred and 11 were nonpreferred, and five tests involved red males and 12 tests involved normal males.

Tests of assumptions

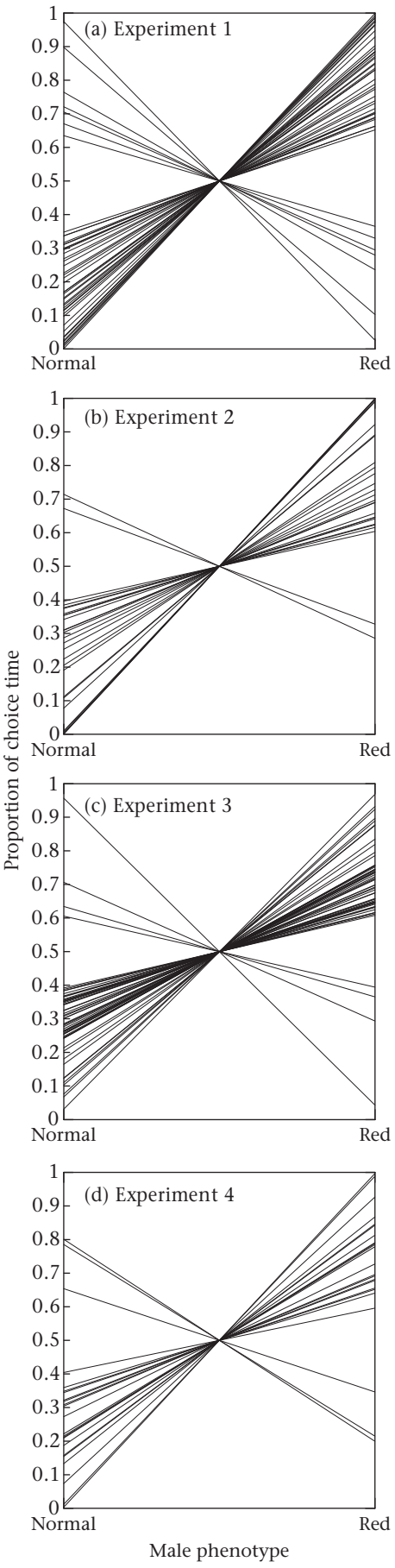
To test the assumption that observed preference patterns reflect mating preferences, we conducted three experiments. (1) Association tests in which females were given the choice of associating with a red or normal female rather than a male after 'lights on'. In this experiment, we tested 34 normal females but eliminated nine trials due to side biases and nine additional trials because test females did not meet the 60% total choice time criterion; thus, 16 females were used in analyses. (2) Association tests in which females were given a choice between a red or normal male; we performed these tests in the afternoon when mating activity is minimal and association is more likely to indicate shoaling preferences. In this experiment, we tested 32 normal females but eliminated three trials due to side biases and nine additional trials because test females did not meet the 60% total choice time criterion; thus, 20 females were used in analyses. (3) Association tests in which females were given the choice between a red or normal female in the afternoon. In this experiment, we tested 24 normal females but eliminated three trials due to side biases and four additional trials because test females did not meet the 60% total choice time criterion; thus, 17 females were used in analyses.

To test the assumption that any preference for red males resulted from a preference for red coloration rather than for UV reflectance, we conducted an association test in which females were given the choice between a red transgenic male and a green transgenic GloFish™ male; these tests were performed immediately after 'lights on'. In this experiment, we tested 37 normal females but eliminated five trials due to side biases and eight additional trials because test females did not meet the 60% total choice time criterion; thus, 24 females were used in analyses.

Statistical Analyses

All analyses were performed using SYSTAT version 10 (SPSS Inc., Chicago, IL, U.S.A.). For all association trial protocols, we used paired *t* tests to determine whether females preferred one male phenotype over the other, based on the percentage of time she spent with each male. All proportional data were angularly transformed. For experiments 1 and 3, we also performed *t* tests to test for differences (1) between females that preferred normal males and females that preferred red males in their preference time with the preferred male, (2) between female phenotypes in total choice time, (3) between female phenotypes in their preference time with the preferred male and (4) between female phenotypes in the amount of total choice time spent in a normal male's choice zone or (5) in a red male's choice zone. We performed multiple regressions to test for the influence of male and female size on each dependent variable of the above tests. For experiments 2 and 3, ANOVAs were used to determine differences in female choice behaviour between rearing treatments.

For the one-male mating tests, we used an ANOVA to determine differences between treatment groups, linear regression to determine the effects of female age and size, and *t* tests to determine differences between red and normal females. We used ANCOVAs to



test for differences in mating latency and the number of times females mated when they were paired with preferred males or nonpreferred males, or with red or normal males. Mating latency times and number of times mated were log-transformed to meet normality assumptions. The model included male type (either preferred or nonpreferred male) or male colour phenotype (either red or normal), the male's prior association time and their interaction. We also performed a chi-square analysis to determine whether females were more likely to mate with one male type (either preferred or nonpreferred male) or male colour phenotype (either red or normal) over the other.

For all tests, $\alpha = 0.05$; in regressions, we considered any regression in which $R^2 < 0.10$ to be biologically nonsignificant, regardless of P value.

RESULTS

Experiment 1

Overall, females associated more with red males than with normal males (average red male preference time: 74.1%, range 2.6–99.9%; paired t test: $t_{46} = 6.84$, $P < 0.001$; Fig. 3a). Females of both colour phenotypes preferred red males to normal males (red females: $t_{23} = 3.85$, $P = 0.001$; normal females: $t_{22} = 6.62$, $P < 0.001$).

Preference time with a preferred male did not differ between females preferring red males or normal males ($t_{45} = 1.24$, $P = 0.22$). Female colour phenotype did not predict differences in total choice time ($t_{45} = 1.70$, $P = 0.10$), preference time with the preferred male ($t_{45} = 1.66$, $P = 0.10$), or the total choice time spent associating with either red males ($t_{45} = 0.05$, $P = 0.96$) or normal males ($t_{45} = 0.70$, $P = 0.49$). Neither male nor female size influenced preference patterns in any test (regression: all $F_{3,42} < 0.89$, all multiple $R^2 < 0.06$, all $P > 0.46$).

Experiment 2

Females in the three populations differing in morph frequency history did not differ in their preference for red males (ANOVA: $F_{2,28} = 1.46$, $P = 0.25$). Overall, females from all three groups associated more with red males than with normal males (average red male preference time: 71.4%, range 28.6–100%; paired t test: $t_{30} = 6.33$, $P < 0.001$; Fig. 3b). When females from different natal populations were tested separately, a consistent preference for red males was also evident (females from the red-morph population: average red male preference time: 74.1%, range 28.6–100%; paired t test: $t_{10} = 3.41$, $P = 0.007$; females from the normal-morph population: average red male preference time: 76.5%, range 59.8–99%; $t_8 = 5.87$, $P < 0.001$; females from the equal-morph population: average red male preference time: 64.5%, range 32.8–92.2%; $t_{10} = 2.87$, $P = 0.017$).

Experiment 3: Association Tests

Overall, females associated more with red males than with normal males (average red male preference time: 69.5%, range 4.1–96.9%; paired t test: $t_{57} = 9.93$, $P < 0.001$; Fig. 3c). Females associated with red males more than normal males in all four

Figure 3. Proportion of choice time (per trial) that each female associated with the normal male and the red male in each experiment: (a) experiment 1: paired t test: $t_{46} = 6.84$, $P < 0.001$; (b) experiment 2: $t_{30} = 6.33$, $P < 0.001$; (c) experiment 3: $t_{57} = 9.93$, $P < 0.001$; (d) experiment 4: $t_{22} = 4.68$, $P < 0.001$. Each line represents one female.

treatment groups (paired t tests: all $t > 3.77$, all $P < 0.04$, $df = 15, 7, 12, 16$, respectively). The five choice behaviours were not influenced by treatment group (ANOVA: all $F_{3,54} < 0.56$, all $P > 0.64$), female age (regression: all $F_{1,56} < 0.04$, all $R^2 < 0.001$, all $P > 0.85$), female size (regression: all $F_{1,56} < 1.60$, all $R^2 < 0.03$, all $P > 0.20$), or female colour phenotype (t test: all $t_{56} < 0.76$, all $P > 0.42$).

One-male Mating Tests

One-male tests provided no evidence for a mating preference, either in terms of latency to mate or in number of mating bouts. Female mating latency times did not differ when females were paired with preferred males or nonpreferred males, or with red males or normal males (ANCOVA with percentage association time as a covariate: main effects: $F_{1,14} < 0.23$, $P > 0.64$; interactions: $F_{1,13} < 1.16$, $P > 0.30$ for both tests). Latency times averaged 903 s (range 119–3157 s). Of the 17 females in this data set, females averaged 10.4 mating bouts (range 2–25 bouts). The number of mating bouts did not differ when females were paired with a preferred male or nonpreferred male, or with a red male or normal male (ANCOVA with percentage association time as a covariate: main effects: $F_{1,14} < 3.13$, $P > 0.10$; interactions: $F_{1,13} < 3.00$, $P > 0.11$ for both tests).

The number of mating bouts per female was unrelated to mating latency (regression: $F_{1,15} = 2.01$, $R^2 = 0.12$, $P = 0.18$). Of the 17 females tested, only six females mated with preferred males, 11 mated with nonpreferred males; and among the 17 females, five mated with red males and 12 mated with normal males; however, these differences were not significant (chi-square test: both $\chi^2_1 < 2.89$, both $P > 0.05$).

The percentage of time males spent chasing females prior to the first mating bout strongly influenced mating latency (regression: $R^2 = 0.52$, $N = 14$, $P = 0.003$; both variables log-transformed; Fig. 4). Red males did not differ from normal males in the percentage of time they spent chasing females (t test: $t_{18} = 0.41$, $P = 0.69$). In contrast, there was no relationship between latency to the first mating bout and the subsequent percentage of time males spent chasing females (regression: $R^2 = 0.22$, $N = 14$, $P = 0.09$; both variables log-transformed). There was no correlation between the percentage of time that males spent chasing females prior and after the first mating bout (regression: $R^2 = 0.09$, $N = 14$, $P = 0.28$; both variables log-transformed).

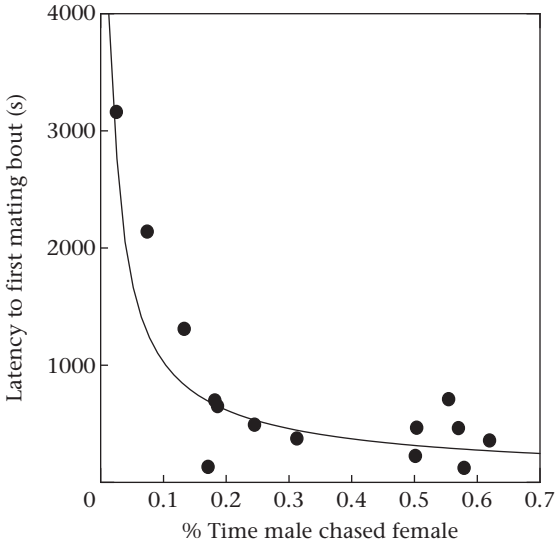


Figure 4. Latency to a female's first mating bout as a function of the percentage of time the male spent chasing the female prior to mating. $R^2 = 0.52$, $P = 0.003$, $N = 14$; both variables log-transformed.

Experiment 4

Overall, females reared on a green diet associated more with red males than with normal males (average red male preference time: 70.4%, range 19.9–99.7%; paired t test: $t_{22} = 4.68$, $P < 0.001$; Fig. 3d). Normal females reared on a green diet showed a strong preference for red males (average red male preference time: 70.8%, range 21.5–99.7%; $t_{15} = 4.11$, $P = 0.001$). Red females showed no preference for red males ($t_6 = 2.15$, $P = 0.08$), but the small sample size severely reduced statistical power, and the result was strongly influenced by one female with a strong preference (80.1%) for a normal male; when we excluded this female from the analysis, all other females strongly preferred red males ($t_5 = 6.28$, $P = 0.002$). When all females were included, red male preference time averaged 69.5% (range 19.9–92.6%).

Tests of Assumptions

In association tests in which females were given the choice of associating with a red or normal female rather than a red or normal male after 'lights on', we found that females had a weaker but still significant association preference for red females (paired t test: $t_{15} = 2.48$, $P = 0.03$). In these tests, however, females showed no preference in nine of the 25 trials conducted that lacked a side bias. In contrast, females that lacked a side bias in experiment 1 showed no preference in only two of the 49 trials (average red male preference time: 63.4%, range 20.5–100.0%; Fisher's exact test: $P = 0.001$). In afternoon tests, females showed no preference for red males (average preference time: 59.3%, range 3.8–99.0%; paired t test: $t_{18} = 1.38$, $P = 0.18$) or red females (average preference time: 57.4%, range 1.6–88.2%; $t_{16} = 1.18$, $P = 0.26$). When given the choice between a red or green GloFish™ male, females preferred to associate with red males (average red male preference time: 64.5%, range 7.4–97.5%; $t_{23} = 3.05$, $P = 0.006$).

DISCUSSION

Of the few published studies of zebrafish mating behaviour to date (Pyron 2003; Darrow & Harris 2004; Spence & Smith 2005, 2006; Snekser et al. 2006), only one has provided support for sexual selection (Pyron 2003), with females preferring larger males.

In all four of our experiments, female zebrafish, regardless of their colour or size, strongly preferred to associate more with genetically modified red males than with normal males. The association preference for red males was evident regardless of the rearing history of the females tested; that is, whether they came from populations with no systematic rearing protocol (experiment 1), from populations that obtained three different morph ratios after six generations (experiment 2), from populations in which sexual imprinting could occur (experiment 3), or from populations fed a diet of only green food (experiment 4).

In our experiments, we assumed that observed association preferences reflected mating preferences rather than shoaling preferences. Our tests of this assumption generally support this view. Although we found a weak, but significant, female association preference for red females in our nonmating association preference test conducted in the morning, we also found more instances of no preference in these trials than in the comparable trials run in experiment 1 in which the test female could choose between two males. In our afternoon tests, when shoaling preferences are expected to be more likely than mating preferences (Hutter et al. 2010), we found no female association preference for red males (or

red females). We interpret both sets of results as indicating that, in our four main experiments, most females were showing a mating preference, but we cannot rule out the possibility that some females may not have been sexually receptive and were showing a shoaling preference. Our afternoon tests may also explain why Snekser et al. (2006) found no female association preference for red males over normal males in zebrafish, because time of day was not controlled in their experiment (S. McRobert, personal communication).

Mate Preference for Novel Male Characters

Mate preference is a key to understanding the rate and direction of sexual selection as it can often provide insight into the evolution of secondary sexual characters (Jennions & Petrie 1997). Preferences for novel male characters are particularly intriguing in that they may suggest how and why preference patterns originate. One possibility is that preferences arise as a result of experiences during ontogeny. Sexual imprinting (Lorenz 1935 cited in Bateson 1978) is a type of ontogenetic learning in which juveniles use members of their social group for cues on species recognition and mate attractiveness. Sexual imprinting has been demonstrated in other fish species including guppies (Breden et al. 1995; Rosenqvist & Houde 1997), sticklebacks (Kozak & Boughman 2009), cichlids (Verzijden & ten Cate 2007) and green swordtails (Walling et al. 2008). However, we found no support for sexual imprinting in our study: females reared in all four social environments associated more with red males than with normal males.

Alternatively, preferences for novel male traits may be influenced by phylogeny. That is, the male character may have been present in ancestors of the species under investigation, but not in the species itself (e.g. the whine-chuck call in *Physalaemus* species, Ryan et al. 1990; forehead ornamentation in auklet species, Jones & Hunter 1998). In other studies, the novel trait may be an exaggeration of an existing trait (e.g. red colour bands on male zebra finches, Burley et al. 1982; increased tail feather length in male widowbirds, Andersson 1982).

The fitness consequences to females that mate with males possessing novel characters might vary considerably among species. Such mating could incur the risk of mating with the wrong species; thus, a complete loss of reproductive success. In contrast, mating with a novel male could provide a benefit of greater offspring genetic heterozygosity or complementarity (Mays & Hill 2004). Hughes et al. (1999) provided female guppies a choice between a male morph with which they had been familiarized and an unfamiliar male morph and found that the novel morph had a higher probability of mating and produced more offspring than the familiar male. Eakley & Houde (2004) also showed that female guppies were more responsive to novel males than to males with whom they had recently mated or to males resembling their recent mate in colour pattern.

Little is known about the proximate mechanisms that underlie female preferences for novel male characters. A sensory bias for red coloration cannot be ruled out as a mechanism for the association preference that we observed. Such a bias could result from the transference of a preference for red coloration used in another context to mating or may persist as a phylogenetic relic. Red coloration of prey increases predation risk by zebrafish (Spence & Smith 2008) and other predatory fish species (e.g. sticklebacks: Ibrahim & Huntingford 1989; Bakker et al. 1997; guppies: Rodd et al. 2002; two-spotted gobies: Utne-Palm 1999). Thus, the visual system of zebrafish (and other fish species) may have evolved a bias to perceive red stimuli selectively as a result of a preference for red-coloured prey. Hence, red-coloured males may appear more attractive. Our experiment 4 provided no support, however, that any transference occurs between the colour of prey eaten and a mate colour preference during ontogeny. Even after being reared on an all-green diet, females retained their preference for red males (experiment 4).

A sensory bias for red males could also reflect retention of a mating preference in female zebrafish for red coloration in males from an ancestral species even though males subsequently lost red pigmentation (Basolo 1990; Ryan et al. 1990). The phylogeny of zebrafish (Fang 2003) reveals several closely related species in which morphs have been described as possessing some degree of red pigmentation, typically some red–orange spots on the lateral surface or on parts of their fins. In contrast, the genetically modified individuals in our study were entirely red. The possibility of an ancestral preference for red coloration could be evaluated experimentally by testing female association preferences of these close relatives of zebrafish as well as by using GloFish™ strains of zebrafish that are green or orange rather than red.

One-male and Two-male Testing Protocols

One-male tests and two-male association tests performed on the same set of females revealed no similarity in patterns of mate preference. In one-male trials, females showed no preference to mate more quickly or more often with a preferred male than with a nonpreferred male or with a red male than with a normal male. The lack of correspondence between the two protocols was not expected, but may be due to behavioural differences that males showed in the one-male tests. Males that spent more time chasing females prior to the first mating bout obtained a mating sooner than males that spent less time chasing females (Fig. 4). Thus, male coercion may have influenced the latencies to mating that we observed.

Each protocol has advantages and disadvantages: the two-male association protocol eliminates male–male competition but may confound mate sampling with mate preference (Wagner 1998). The one-male mating protocol eliminates male sampling as well as

Table 2
Studies using both a simultaneous two-male testing protocol and a no-choice, one-male testing protocol

Species	Male phenotype	Stimulus	Same females	Preference assessment	Two protocols	Source
Drosophila simulans	Ebony coloration	Males	No	Mating latency	Similar preferences	Sharma et al. 2010
Bush crickets	Body size	Males	Yes	Phonotaxis/Mating latency	Phonotaxis: preference for larger male Latency: no preference	Lehmann & Lehmann 2008
Zebra finch	Wild or domesticated	Males	Yes	Association time/ Female response	Opposing results in the two protocols	Rutstein et al. 2007
Wolf spiders	Leg tuft size	Videos	No	Receptivity display	Results differed, but in both tests, female response varied with reproductive status	Uetz & Norton 2007
Wood crickets	Male calls	Playback	No	Phonotaxis	Similar discrimination patterns	Jang & Gerhardt 2006
Tree frogs	Male calls	Playback	No	Phonotaxis	Similar patterns	Bush et al. 2002
Sailfin mollies	Body size	Scanned images	Yes	Association time	Stronger pattern in simultaneous tests	MacLaren & Rowland 2006

male competition, but mating activity could be influenced by male behaviour (i.e. mate coercion) in addition to (or instead of) female preference. Therefore, we subjected the same females to both preference assessment protocols in experiment 3 to compare results obtained by the two methods.

As Wagner (1998) noted, most tests of mating preferences use a simultaneous testing protocol involving at least two males. He argued in favour of single-male tests, however, because simultaneous trials could confound mating preference with mate sampling. Our review of 127 papers on mate preferences published after 1998 that cited Wagner (1998) revealed that simultaneous testing is still the predominant protocol: 91 studies (71.7%) used a simultaneous protocol, 29 studies (22.8%) used a one-male protocol, and seven studies (5.5%) used both protocols. Of the latter seven studies, four found a correspondence in the results obtained using the two protocols and three did not (Table 2). Although sample size is small, the two protocols in these seven studies seem more likely to be in agreement when the stimulus for choice provided to the females is some male attribute (i.e. a vocalization or image) rather than the males themselves. Thus, male behaviour may reduce the effectiveness of one-male tests in assessing female preference. In addition, only three of the seven studies used both protocols on the same females, thereby allowing a more direct comparison. In all three studies, stronger responses were typically observed when using a two-male protocol.

Coda

The use of genetically modified strains has proved to be an invaluable tool in studies investigating genetics and development in an array of species; however, this technology is under-utilized in tests of evolutionary hypotheses. To date, a few studies have used genetically modified strains to study mate preference, but the potential of this technology has scarcely been tapped. Avenues for future research include inserting genes that confer disease or parasite resistance that are linked to genes that influence expression of an indicator trait (Andersson 1994) to test 'good genes' models, or inserting genes that not only affect the expression of a sexual trait but also inflict a viability cost to test predictions of runaway models (Fisher 1930; Lande 1981; Kirkpatrick 1982). Combining genetic modification tools with quantitative trail loci (QTL) analyses that may find genes affecting both male secondary sexual trait expression and female preference could issue in a new age of discovery in evolutionary biology by allowing tests of predictions that heretofore seemed impossible.

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