Phenotypic differentiation and pre-mating isolation between allopatric populations of *Girardinichthys multiradiatus*

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Sexual selection may lead to reproductive isolation between populations through divergence in female mate choice, and population differentiation driven by female mate choice is expected to produce pre- but not post-mating isolation. We tested these hypotheses by looking at whether allopatric populations of the Amarillo (*Girardinichthys multiradiatus*), a sexually dimorphic viviparous fish with effective female choice, (i) have undergone phenotypic differentiation that may be attributed to divergence in female mate choice, and (ii) are already separated by pre- and/or post-mating reproductive barriers. We found substantial divergence in morphological traits which are the target of female mate choice, and in male courtship behaviour. Strong female preferences for homogametic males indicate substantial and symmetric pre-copulatory isolation, but the few successful heterogametic crosses produced in confinement yielded litters of the same size as those produced in homogametic matings, suggesting that post-copulatory isolation between populations is non-existent or weak. It appears that the studied populations have undergone incipient speciation with a pattern that is consistent with speciation driven by sexual selection, yet further work should assess whether divergence in female preferences has promoted male phenotypic differentiation or whether variation in male attributes has driven divergence in female preferences.

Keywords: *Girardinichthys multiradiatus*; reproductive isolation; sexual selection; pre-mating isolation; population differentiation

1. INTRODUCTION

Species remain distinct because there are barriers that prevent gene flow between them (Dobzhansky 1937). In contrast to extrinsic barriers such as geographic discontinuity, which can be ephemeral, post-zygotic and behavioural (mostly pre-zygotic; Butlin & Ritchie 1994) barriers are diagnostic of the specific status of related organisms. Post-zygotic barriers are thought to result from the accumulation through time of allelic (Dobzhansky–Muller, or M–D) incompatibilities between isolated populations (Dobzhansky 1937; Muller 1942; Orr 1995). Behavioural barriers to gene flow are thought to evolve more rapidly. These often involve a suite of signals and responses known as the Specific Mate Recognition System (SMRS; Paterson 1985). Since sexual selection frequently acts on the same characters involved in the SMRS, it has been considered as a sufficiently powerful force to generate reproductive isolation (Panhuis *et al.* 2001). Furthermore, as traits involved in sexual selection can diverge quickly and with little genetic differentiation (Coyne 1994; Schausen & Van Alphen 1998; Uy & Borgia 2000), and given that it frequently operates before genetic incompatibilities may be expressed, sexual selection is expected to promote rapid speciation (Panhuis *et al.* 2001), but demonstrations that this process creates effective pre-mating barriers are scarce (see Magurran 1998). Here, we sought evidence that sexual selection promotes interpopulation differentiation and pre-mating isolation in a fish with effective female choice.

Girardinichthys multiradiatus is a viviparous fish from the Mexican plateau. Males possess big and colourful fins which make them vulnerable to snake predation (Macías García *et al.* 1994, 1998), and display conspicuous courtship behaviour. Both male morphology and behaviour are the targets of female mate choice (Macías García *et al.* 1994; Macías García & Saborío 2004), and the lack of an intromittent gonopodium means that males cannot circumvent female mate choice because internal insemination requires female cooperation (Macías García & Saborío 2004). As a result of a complex geomorphological history (Webb *et al.* 2004; Gesundheit & Macías García 2005), the distribution of *G. multiradiatus* has been fragmented and populations have had the opportunity to diverge. This species therefore constitutes a good model to investigate the hypothesis of rapid population divergence led by sexual selection.

2. MATERIAL AND METHODS

We used wild-caught fish from five isolated localities selected to represent the complete geographic range of the Amarillo fish. Santiago and Salazar, in the upper Lerma basin, lie at the core of this species' distribution; Porvenir and Zempoala represent two of the three groups of populations in the Balsas drainage, and San Juanico represents one of the two tributaries of the Tula (Pánuco drainage) which are occupied by *G. multiradiatus* (figure 1a).

(a) Morphological variation

Between nine and 31 males ($\bar{x}$ = 17, s.d. = 8.7) and 10–18 females ($\bar{x}$ = 14.4, s.d. = 3.3) of each population were

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Figure 1. (a) Hydrographic map of Central Mexico showing the distribution of *Girardinichthys multiradiatus* (in gray) and the watershed divides (black lines). Most populations are found in the upper River Lerma basin (UL), but some inhabit localities in the upper reaches of a few tributaries of the Balsas (B) and Pánuco (P) basins (M=approximate location of Mexico City conurbation). (b) Male body depth (1) total length (2; an average of 3.98 cm in this study) and dorsal and anal fin morphology (3–6) have been shown to influence female mate choice. We also measured eye (A) and pupil (B) diameter, as well as caudal peduncle height (D) and caudal fin length (C) as examples of attributes presumably unrelated to sexual selection (see §3). The reduced black silhouette of a female is shown for comparison (both drawings based on fish from Zempoala).

anaesthetized with drops of 1 g benzocaine/100 ml acetone, and digital pictures were taken under standardized conditions. Using the software IMAGE TOOL, we measured six attributes related to body and fin shape, which have

previously been shown to be targets of intersexual selection in this species (Macías García *et al.* 1994; Macías García *et al.* 1998) and four attributes which have not been implicated in intersexual selection (figure 1b).

(b) Behavioural variation

Male behaviour towards females from their own population was quantified. A female was placed in a plastic bag and introduced into the experimental tank. A male was then introduced and after 15 min the female was released and the trial began, recording the duration and frequency of male displays (lateral fin display, frontal fin display, courtship fin folding, flagging and figure-of-eight dance; Macías García 1991). This was repeated for 8–12 males ($\bar{x}=10.4$, s.d.=1.28) from each population, each exposed only to one female. These 52 males and females were used only once in this experiment.

(c) Female mating preferences

Between 20 and 25 females from each population ($\bar{x}=22.6$, s.d.=1.7) were exposed to a male of their own and a male from one of the other populations (5–7 replicates per combination; $\bar{x}=5.65$, s.d.=0.52). We placed two males in plastic bags at opposite ends of a 40 l tank during 20 min trials. After 10 min, a female was introduced into the tank and the time that she remained at a distance of less than one body-length from each male was measured, which was facilitated by the fact that males always move towards approaching females. We also recorded the frequency of copulation attempts (mutual embraces of male and female through the plastic bag). Mate choice in these conditions is probably based on male morphology and, to a lesser degree, on behaviour (since males in bags cannot fully perform courtship displays). Dorsal fin colour (yellow in males) may have influenced sexual preference, but some wavelengths such as UV, which are used in mate choice (Macías García & Burt de Perera 2002), were not present. Chemical information was also not available.

(d) Post-mating isolation

We placed in an experimental tank one male and one female which had recently given birth in isolation, and left them undisturbed for 20 days. Then the male was removed and the female remained in the tank for a further two months (gestation in captivity = 53 ± 7.9 days, $n=13$; E. Saborio and Macías García, unpublished data), when pregnancy was scored. Pregnancy was the result of mating with the provided male, as Goodeid females do not store sperm for consecutive broods. Five pairs were formed of each of the 25 combinations.

3. RESULTS**(a) Morphological variation**

A principal component analysis was performed to quantify the inter-population divergence in morphology and to assess whether traits involved in sexual selection are more divergent than other morphological traits. This analysis used 157 fish from both sexes and five populations. The first component (48.2% of variance explained) assigned large (absolute) loadings to the variables that have been implicated in sexual selection ($\bar{x}=0.385 \pm 0.057$, $n=6$), and low loadings to the eye (0.168 and 0.255) and caudal (0.041 and 0.002) measurements (see figure 1b). The second component (17.2%) gave more weight to the caudal morphology (0.684 and 0.693) than to that of the eye (0.056 and 0.015) or to morphological attributes reportedly involved in female mate choice ($\bar{x}=0.0702 \pm 0.062$). Males and females were readily

differentiated along the dimension of PC1, but not of PC2 (figure 2a). That PC1 is a measure of sexual dimorphism in body shape was confirmed by an ANOVA ($F_{1,156}=331.05$, $p=0.000$), which also revealed significant differences among populations ($F_{4,156}=4.56$, $p=0.002$), as well as a significant sex-by-population interaction ($F_{4,156}=3.25$, $p=0.014$). *Post hoc* comparisons showed that some male populations were significantly different, but there were no differences between female populations (figure 2a). All of the per-population comparisons between sexes were significant.

If population differentiation were only the consequence of local differences in naturally selected morphology, male and female morphology should evolve in similar directions and at similar pace (in the absence of evidence of niche partitioning between sexes in *G. multiradiatus*; see Macías García 1994). We found that population differences in the sexually dimorphic PC1 co-varied between sexes, but a slope of greater than one suggests that male morphology is evolving more rapidly than female morphology ($r=0.59$, Mantel test, $p=0.007$; figure 2b). This inference is supported by the fact that the average absolute inter-male population difference (i.e. its magnitude, regardless of the direction of the difference; $\bar{x}=0.907 \pm 0.610$) was larger than the average difference between female populations ($\bar{x}=0.412 \pm 0.338$; paired *t*-test = 2.41, $p=0.039$, $n=10$). Male and female population differences in PC2 scores were almost perfectly concordant ($r=0.96$, Mantel test, $p=0.014$; figure 2c) and of equal magnitude (males, $\bar{x}=0.946 \pm 0.562$; females, $\bar{x}=0.896 \pm 0.539$; paired *t*-test = 0.49, $p=0.635$, $n=10$), as expected in the case of attributes which are not the target of sexual selection.

(b) Behavioural variation

The 10 recorded behavioural variables were subjected to a principal component analysis. The first principal component (PC1) explained 41.8% of the variance and gave large loadings to high-intensity courtship behaviours (i.e. flagging and figure-of-eight dance; Macías García & Saborio 2004), and the second principal component (PC2; 23.4% variance explained) gave more weight to relatively static postural displays (e.g. lateral fin display). There were substantial differences between populations in both PC1 ($F_{4,47}=5.66$, $p<0.001$) and PC2 ($F_{4,47}=5.66$, $p<0.001$; figure 3).

(c) Female mating preferences

Females of all populations spent more time with the homogametic ($\bar{x}=235.3 \pm 22.5$ s, $n=20$ contrasts) than with the heterogametic males ($\bar{x}=140.8 \pm 33.3$ s). This preference was significant in 16 out of the 20 contrasts (figure 4a), and a combined analysis using data from each population only once (i.e. contrasting the mean proportion of time with homogametic versus time with heterogametic males without separating these by population) was highly significant ($t=12.5$, d.f.=8, $p<0.0001$). The proportion of time spent with homogametic males ($\bar{x}=63.2 \pm 5.7$, range 52.6–76%, $n=20$) varied symmetrically, with reciprocal trials showing similar degrees of homogametic preference (figure 4b). Time next to a male reflects female mating preferences; 53 out of the 61 female copulation attempts were with homogametic males ($\chi^2=33.2$, d.f.=1, $p<0.0001$) and the two measures

Figure 2. (a) Sexual dimorphism in body shape is captured by the first principal component (48.2% of variance explained), which differentiates between sexes and among male, but not among female populations (different letters indicate significant contrasts). (b) Although, sexes co-vary, male populations are more differentiated than female populations in their mean PC1 scores (sexual dimorphism). (c) Population divergence in non-sexually selected traits (PC2; 17.2% of variance explained) is the same for both sexes.

(tendency to copulate with homogametic male and time next to it) were significantly correlated (ordinal logistic regression; $Z = -2.66$, $p = 0.008$, $n = 113$ females).

(d) Post-mating isolation

Fourteen (56%) of the 25 females confined with homogametic males became pregnant, whereas only 24 of the 100 females confined with heterogametic males did ($\chi^2 = 8.23$, d.f. = 1, $p = 0.002$). This pattern of differential fecundity was shared by most populations, although there was some variance between populations (homogametic mating success $\bar{x} = 0.56 \pm 0.22$, heterogametic $\bar{x} = 0.24 \pm 0.17$, t -test on arcsine-transformed data, $t = 2.81$, d.f. = 4, $p = 0.048$). Fecundity (= brood size), however, was not different between homogametic ($\bar{x} = 18.2 \pm 4.3$) and heterogametic pairs ($\bar{x} = 16.2 \pm 7.3$; $t = 0.5$, d.f. = 6, $p > 0.63$).

4. DISCUSSION

A way to detect the action of the sexual selection is to quantify the magnitude of sexual dimorphism (Barraclough *et al.* 1995). We verified that sexual dimorphism, as measured by a canonical variable (PC1) largely influenced by traits that have been implicated in female mate choice (Macías García *et al.* 1994, 1998,

Figure 3. (a) We reduced the number of behavioural variables to two principal components, which together explain 65.2% variance in courtship. (b) Significant differences between populations (indicated with different letters) were not the same in both components, suggesting that behavioural differences are not simply the consequence of some populations being more and some less active during courtship.

Figure 4. (a) Bars represent the mean ($\pm$ s.d.) time spent by 5–7 females of the population indicated by the first letter in the left column (i.e. Zempoala in the first four rows) with the homogametic (left bars; note that values are positive in both directions) or the heterogametic male (SJ=San Juanico, Sgo=Santiago, Sa=Salazar and P=Porvenir) during 10 min trials. In all combinations, females spent more time with the homogametic male, but the difference was not always significant (paired t -tests; one, two and three asterisks denote p -values less than 0.05, 0.01 and 0.001, respectively). (b) The mean proportion of time with homogametic males was averaged for all the females of each population. This measure of the intensity of pre-mating isolation was symmetric, with female discrimination being correlated in reciprocal tests.

Macías García & Saborío 2004) is substantial in the Amarillo fish, and that it varies considerably between populations. We further found that variation in sexually selected morphology is greater than variation in non-sexually selected morphology and that male morphology diverged faster among populations than female morphology (this may also apply to colour, which we did not measure, since only males have yellow fins, which are the target of female choice and appear to vary in brightness between populations). These findings are consistent with the hypothesis that sexual selection drives population differentiation in this fish (Panhuis *et al.* 2001).

Population differences were not confined to body shape. Males from the five populations performed the same courtship displays, but varied markedly in their tendency to use them. Our data confirm that at least some aspects of the sexual behaviour of *G. multiradiatus* are substantially different between populations (Macías García 1994). Since we used wild-caught fish, it is possible that these differences reflect exposure to different environments before capture. We argue that this is not the case because the behaviour of fish from different populations remains distinctive in aquaria through life even if the fish are very young when captured (C. Macías García,

personal observation), and show significant additive genetic variance (Berea de la Rosa and Macías García, unpublished data). If congenital, the observed differences may be the result of local variation in the selective forces that constrain the expression of epigamic characters (with or without the involvement of differences in female mate choice), random population divergence in female choice, or both. We have identified one geographically variable constraint on ornament elaboration in *G. multiradiatus*: the prevalence of its main predator (the snake *Thamnophis melanogaster*; Macías García 1994; Macías García *et al.* 1994, 1998). Since we lack appropriate snake censuses we cannot at present determine whether variation in ecological constraints or random differences in female choice account for the observed population differentiation. We now know that female mate choice is involved.

Females from all localities demonstrated a strong predisposition to mate with homogametic males. This has been shown in other species (Endler & Houde 1995; Herring & Verrell 1996; Ritchie 1996), but in addition we found that the strength of the female preference for homogametic males was symmetrical, with females of a given population being as discriminating in favour of their own males when presented alongside males of another locality, as were females from the latter population when the reciprocal test was applied. This pattern implies that female *G. multiradiatus* do not have open-ended preference functions, since that would produce asymmetries in female homogametic preference as males from populations where ornaments are more developed would be preferred by females from their own as well as from other populations. The observed pattern rather suggests that female Amarillo fish have convex mating preference functions that are not completely overlapped between populations, probably due to local differences in the costs of displaying the male ornaments. This inference should be experimentally verified. It must also be experimentally verified to what extent female preferences translate into mating isolation in the presence of male–male competition (Macías García 1994).

Females given access to only one male were more likely to become pregnant if the male was homogametic than if it was heterogametic. This pattern has four possible explanations: (i) females copulated with both types of male but the capacity of heterogametic sperm to inseminate ova was reduced; (ii) both copulation rate and fecundity were similar between homogametic and heterogametic pairs, but heterogametic embryos were less viable; (iii) females copulated only with males that were genetically compatible, which was more likely to occur in homogametic pairs; or (iv) fertility was the same between homogametic and heterogametic pairs, but females refused more often to mate with heterogametic than with homogametic males. Some pairs of each combination were fertile, thus heterogametic sperm appears capable of inseminating ova (explanation (i), above). Litter size was not different between the two types of pair, therefore heterogametic embryos are not necessarily unviable (ii). We cannot rule-out the possibility that females can detect post-zygotic compatibility with a male prior to copulation (iii), but a more parsimonious explanation given the results of the female-choice tests (and since female *G. multiradiatus* can avoid unwanted copulations; Macías García & Saborío 2004) is that females were more likely to

mate with homogametic males (iv), and that no obvious post-copulatory barriers exist between populations. We propose that sexual selection through diverging female mate choice has promoted the evolution of pre- but not post-mating barriers in *G. multiradiatus*, and that these have led to population differentiation in both the extent of sexual dimorphism and in the style of male courtship displays, thus constituting a case of incipient speciation.

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