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Absence of female preference and the origin of a unisexual species, the Amazon molly (*Poecilia formosa*)

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Abstract

The role of hybridization as a formative process in evolution has received much attention in the past few decades. A particularly fascinating outcome of hybrid speciation is the formation of asexual hybrid species. The Amazon molly (*Poecilia formosa*) is such a hybrid and originated from a *P. mexicana* mother and a *P. latipinna* father. Consequently, a heterospecific mating must have occurred leading to the Amazon molly, indicating a breakdown of any potential prezygotic isolation between parental species. Here we studied the female mate preferences of extant *P. mexicana* and *P. latipinna* from several populations using standard binary choice tests with males of both sexual species that were matched for size. *Poecilia mexicana* and *P. latipinna* can be crossed in the lab, however, the offspring are not asexual, but sexual F_1s. In our study, we generated F_1s and tested their mating preferences with sexual males of both *P. mexicana* and *P. latipinna* against F_1 males. Overall, our results show that in extant *P. mexicana* and *P. latipinna* no female preference for conspecific males was detectable. Consequently, heterospecific matings are possible and not hindered by any apparent behavioral prezygotic isolation. If female preferences in these species were comparable around the time the Amazon molly originated as a hybrid species ca. 100,000 years ago, matings leading to hybrids would be very likely. F_1 females also have no discernable mating preferences for either sexual males or F_1 males. Such lack of prezygotic behavioral isolation could potentially lead to F_2 individuals, backcrosses, and introgression.

KEYWORDS
asexual, hybridization, mate choice, Poeciliid, stock center

1 | INTRODUCTION

Hybridization between species in nature implies the absence or breakdown of reproductive isolating mechanisms (Rieseberg et al., 2000). These isolating mechanisms are thought to have evolved in response to costly hybridizations, especially for females. They are broadly classified into (i) mechanisms that prevent mating between two different species (prezygotic isolation) (Dagilis & Kirkpatrick, 2016) and (ii) mechanisms leading to reduced fitness of hybrid offspring (postzygotic isolation) (Presgraves, 2002). Hybridization is recognized as an important factor in the evolution of species (Moran et al., 2021). Often hybridization leads to introgression that is best detectable by genetic methods (Cui et al., 2013; Noonan et al., 2006; Powell et al., 2020). Full hybrid speciation, when hybrids are reproductively isolated from their parental species (Abbott et al., 2013; Runemark et al., 2019) appears to be much rarer, raising the question of *why* hybrid species are so rare (or transient on an evolutionary time scale) when hybrid matings must be quite common. Alternatively, hybrid species may be common but transient, therefore going undetected (Janko et al., 2018), partly because scientists are not systematically looking for them. In many cases, hybrid species show significant deviations from sexual reproduction, typically some form of clonal reproduction (Avise, 2008; Schlupp, 2005). These hybrid species with new reproductive modes are of particular interest to understand the evolutionary pathways that lead to the transition from sexuality to asexuality, as many asexual eukaryotes have been shown to be of hybrid origin (Suomalainen et al., 1987). In general, the resulting asexual modes of reproduction are genetically totally (parthenogenesis and gynogenesis) or partially (hybridogenesis) clonal, involve unreduced eggs, and sometimes require sperm to trigger embryogenesis (Avise, 2008; Suomalainen et al., 1987). The evolutionary pathway to emergence of these asexual species is still highly debated: though it is often thought that asexuality appears readily as a direct and immediate result of hybridization (Schlupp, 2005), it has been proposed that asexuality can evolve from sexual hybrids through multiple small and incremental evolutionary steps (Fyon et al., 2023).

Several hypotheses to explain the rarity of asexual hybrids have been proposed. Moritz et al. (1989) suggested the "balance hypothesis," postulating that asexuality should emerge within a certain range of genetic divergence between parental species. Within that range, the accumulation of incompatibilities between parentals is enough to disrupt meiosis, but not viability and fertility in their hybrids (Moritz et al., 1989). Such a pattern was recently found in Whiptail lizards (Barley et al., 2021, 2022). Using the Amazon molly (*Poecilia formosa*) as example, Stöck et al. (2010) proposed the "rare formation hypothesis" (or lottery hypothesis), suggesting that asexual hybrids are rare not due to inherent disadvantages of asexuality, but because a very specific genomic combination is needed to allow hybrid viability and clonal reproduction (Stöck et al., 2010; Warren et al., 2018). It should be noted that in species that are sperm dependent (all asexual hybrid fishes and amphibians), the ecology of their establishment and maintenance is particularly complicated to explain in an eco-evolutionary framework, given their need to coexist with (but not outcompete) their sexual sperm donors, among other effects (Cerepaka & Schlupp, 2023; Janko et al., 2023; Riesch et al., 2012).

Once established, asexual hybrids play important ecological roles, for example, via resource competition with coexisting sexual species, or even in speciation of sexual species (Janko et al., 2023; Vrijenhoek, 1994). Asexual Amazon mollies (*P. formosa*) show almost complete niche overlap with their sexual hosts, for example, when it comes to feeding (Scharnweber, Plath, & Tobler, 2011; Scharnweber, Plath, Winemiller, et al., 2011), or parasite load (Tobler & Schlupp, 2005). In the present study, we use the Amazon molly (*P. formosa*), a unisexual hybrid species to investigate the role of mate choice in the formation of hybrid species. Hybridizations leading to unisexual species often happened a long time ago (Avise, 2008) (in the case of the Amazon molly, at least 100,000 years; Schartl et al., 1995; Warren et al., 2018) and any role behavior may have played in the formation of hybrids may be shrouded in history. In the case of the Amazon molly, however, the parental species are known and extant, allowing for experiments that provide an approximation of the original hybridization. We are trying to shed light on a particularly poorly understood aspect of the origin of hybrid species and hybridization, in general, namely the role of behavior. The study of female mate choice is particularly relevant for the origin of unisexual Amazon molly (*P. formosa*), which originated via a hybridization event (Lampert & Schartl, 2008; Schlupp, 2005) between a *P. mexicana* "mother" and a *P. latipinna* "father" (Hubbs & Hubbs, 1932). The hybrid origin of Amazon mollies has been confirmed several times through molecular work (Avise et al., 1991; Schartl et al., 1995; Tiedemann et al., 2005; Warren et al., 2018). The Amazon molly is characterized by several uncommon traits. In addition to being a hybrid species, Amazon mollies have no functional meiosis and produce unreduced, diploid eggs through apomixis (Dedukh et al., 2022; Monaco et al., 1984). They are clonal, all-female, but require sperm to trigger embryogenesis. The two parental species that were involved in the single original hybridization that led to the Amazon molly have been identified and are extant, with the Atlantic molly *P. mexicana* (female parent) being distributed from South to Central Mexico, while the Sailfin molly *P. latipinna* (male parent) is present in coastal environments of the Gulf of Mexico and the Southeastern USA (Schlupp et al., 2002) (Figure 1). These two species are also the main sperm donors for Amazon mollies.

Clearly, a hybrid species can only arise if heterospecific matings occur. This, however, is usually predicted to be selected against, but only if hybrid individuals are less viable or fertile; interestingly, theory suggests that assortative mating can be a relatively weak force in promoting speciation (Irwin, 2020). There are examples, however, of adaptive heterospecific preference (Mendelson & Shaw, 2012; Pfennig, 2007; Ryan & Wagner, 1987), sometimes governed by sensory bias (Basolo & Endler, 1995; Ryan, 1990). One reason why this problem may be difficult to address is that the parental species of a hybrid are often either unknown (Suomalainen et al., 1987), extinct (Freitas et al., 2019, 2022), or otherwise unavailable. In a few cases, however, the parental species are known and extant. Only in such cases can we experimentally study the prerequisites for hybridization, namely mate choice for conspecific species. Of course, even when we have access to the parental species, one limitation is that the parental species have evolved between the time of hybridization that led to an asexual species and present time (Dries, 2003; Makowicz & Travis, 2020). Nonetheless, a study like the one presented here is important to shed light on the origin of unisexual hybrids. In the following, we ask whether females from two livebearing fishes from the family Poeciliidae (and their F_1 hybrids) with partially overlapping habitats show species preference as a prezygotic isolating mechanism.

Furthermore, sympatry and syntopy are prerequisites for heterospecific matings to occur. Thus, for a meaningful test of mating preferences of the extant species, they should also overlap. Both of these conditions are met in the Amazon molly. A detailed analysis

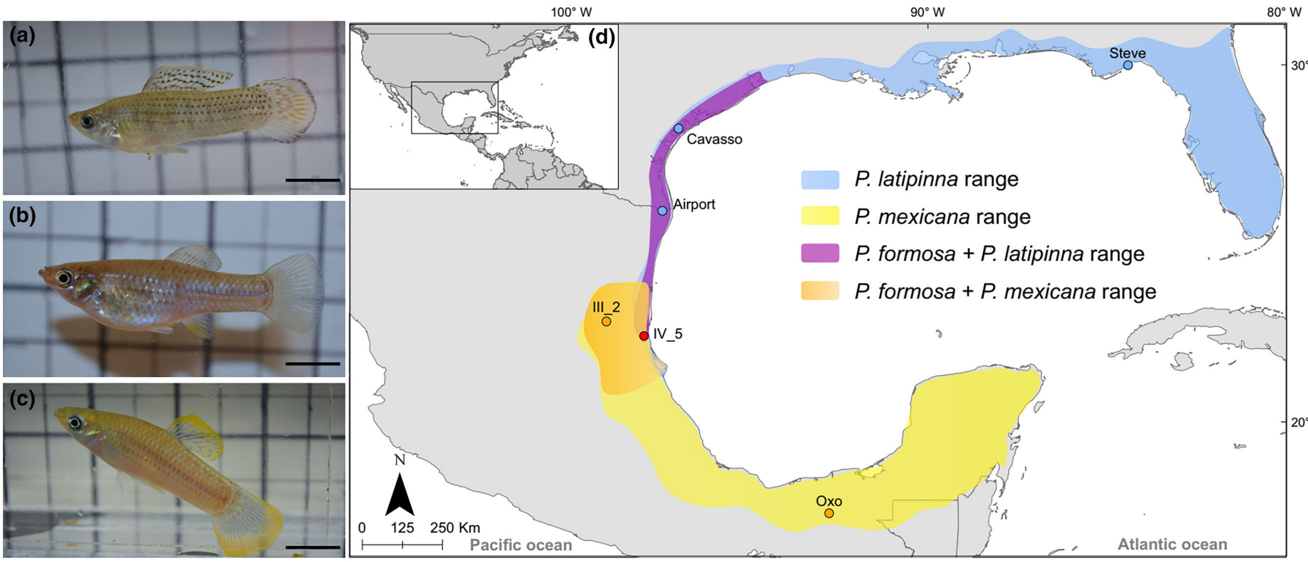

FIGURE 1 Males of *Poecilia latipinna* (a), *Poecilia mexicana* (c), and a female F_1 (b). The thick black bar in the lower right corner is a scale of 1 cm. The map depicts the current ranges of *P. formosa* (orange and purple), *P. mexicana* (yellow), and *P. latipinna* (blue). In the orange area, *P. formosa* coexists with *P. mexicana*, in the purple area, it coexists with *P. latipinna*. The area of current sympatry of all three species is limited to the area around IV/5 (dark red). Circles indicate the populations used; note that we used populations from sympatry and allopatry. The insert (d) shows the area of the detailed map. See text for details.

of mitochondrial haplotypes revealed that an area near Tampico, Mexico is the most likely place of origin of the Amazon molly (Stöck et al., 2010). This was independently supported by environmental niche modeling of the two parental species looking back in time (Costa & Schlupp, 2020). Interestingly, this is also one of the few areas, where both *P. mexicana* and *P. latipinna* currently coexist and are syntopic. This makes a particular population from a lagoon near Tampico, Mexico, a prime candidate for investigating the origin of Amazon mollies. It has already been used successfully in crossing *P. mexicana* and *P. latipinna* (Berbel-Filho & Schlupp, unpublished data; Lampert et al., 2007), in addition to other populations (Dries, 2003; Hubbs, 1934, 1946a, 1946b; Makowicz & Travis, 2020; Turner, Brett, & Miller, 1980).

Mating preferences between parental species can direct the evolutionary outcomes of hybridization. In the Amazon molly system, what is unknown so far is how likely the original hybridization would be with current individuals of *P. mexicana* and *P. latipinna*, especially from the syntopic populations from Tampico. The existence of the Amazon molly indicates that a unidirectional mating happened at least once, but how likely is it? Furthermore, why did the hybridization lead to Amazon mollies in only one direction? Previous attempts to cross the parental species are not informative here, because they were set up as forced crosses of one or several unmated (virgin) female(s) and one or several males, leaving little opportunity for females to exercise mate choice. In general, mate choice in this system has been addressed widely (Schlupp, 2005, 2009, 2018), including character displacement in males (Gabor et al., 2005; Gabor & Ryan, 2001). Many hypotheses exist that can explain female preferences, including an almost universal preference for larger males size (Ryan & Keddy-Hector, 1992), but in the context of this study, the idea of a preexisting bias governing mate preferences of female mollies, in particular females of species with short dorsal fins (Ptacek, 1998) because males are more ornamented, seems particularly relevant (Endler & Basolo, 1998; Ryan, 1990) (Figure 1). For example, MacLaren and Rowland (2006) studied *P. mexicana* females from Rio Verde (likely from sympatry with *P. formosa*) and found that they prefer larger males (see also MacLaren et al., 2004), predicting heterospecific matings of such females with *P. latipinna* males. Note, however, that no Sailfin mollies are presently known from that area. Ptacek (1998) made similar observations for another shortfin molly species, *Poecilia orri*. In a field study on *P. mexicana* and *P. velifera*, a close relative of *P. latipinna*, *P. mexicana* males showed strong sexual interest in *P. velifera* females (Domínguez-Castanedo & Schlupp, 2023). If such preferences can be generalized, therefore, for the particular case of the origin of Amazon mollies this hypothesis predicts that a short fin molly female, such as *P. mexicana*, would be likely to mate with a long-fin molly male, such as *P. latipinna*. In addition, this hypothesis predicts a directionality of matings that is in agreement with the actual hybridization event led to the Amazon molly. However, a lack of preference and random mating by *P. mexicana* would also lead to hybridization, and the absence of asexual de novo hybrids in the wild with a long-fin molly mother may be explained by unidirectional or sex-linked Muller-Dobzhansky incompatibilities (Schlupp, 2005). Yet, both hypotheses, mating based on sensory bias, and random mating fail to account for the virtual absence of hybrids in nature. Asexual *de novo* hybrids are also unknown from laboratory crosses; instead all attempts to generate *de novo* asexual hybrids have resulted in sexual F_1's (e.g., Berbel-Filho & Schlupp, unpublished data; Dries, 2003; Hubbs, 1934; Lampert et al., 2007; Makowicz & Travis, 2020; Turner, Brett, & Miller, 1980). Therefore, in our study, for the first time, using a large data set from multiple populations, we ask if females of *P. mexicana* and *P. latipinna* prefer conspecific males over heterospecific males. Furthermore, we study the mate preferences of a small number of F_1 females and ask if they have a preference for males of the parental species.

Because heterospecific matings are generally considered to be costly, we predict that females should prefer conspecific males. To address this, we used standard binary choice tests (Figure 2) and multiple populations of each species to account for within-species geographic variability (Powell & Schlupp, 2024).

Furthermore, we used unmated (virgin) and previously mated females in *P. mexicana* to elucidate the potential role of previous (or absence of) mating experience (Richardson & Zuk, 2023). For this particular situation, we predicted stronger preferences in females that had been together with conspecific males because individual experience might reinforce their preference and the mated females had been together only with conspecifics.

In a subset of our experiments, we deeply explored the question of how female preferences may be relevant to hybridization. We know that hybridizations leading to sexual F_1 happen—but apparently only in the laboratory (e.g., Berbel-Filho & Schlupp, unpublished data; Dries, 2003; Hubbs, 1934; Lampert et al., 2007; Makowicz & Travis, 2020; Turner, Brett, & Miller, 1980). On the other hand, a hybridization leading to an asexual also happened in nature in the past, as evidenced by the existence of Amazon mollies. In other words, at some point in time, likely F_1 females (possibly sexual, as in laboratory crosses) were faced with a choice between males of one or both of the two sexual species *P. latipinna* and *P. mexicana* and F_1 males. For the first time to our knowledge, we were able to study female mate preferences in this situation. Male F_1s have been studied by Dries (2003). She tested if males of *P. latipinna* and *P. mexicana* would prefer F_1 hybrid females over gynogenetic Amazon mollies but found no differences. For our study, we would predict F_1 females to prefer F_1 males over sexual males if phenotypic similarity is a factor in mate preference.

Finally, we were interested in the role of side biases. This is a common phenomenon in studies using binary choice tests. This occurs when a chooser spends all (or most) of their time on one side

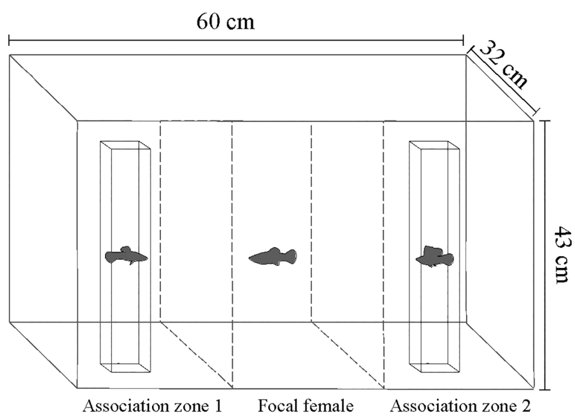

FIGURE 2 Schematic of the choice tank with dimensions.

of the choice arena, without following the preferred stimulus during the customary side switching of the stimuli. In previous work, we have already used a priori exclusion rules (Schlupp et al., 1994; Schlüter et al., 1998). Side biases may be caused by a number of factors, such as a general lack of motivation to choose and problems with the design of the choice apparatus. The latter should result in directional side biases, where a majority of the side biases are to one side only. However, side biases may also indicate that the choosers have trouble discerning the two alternatives presented or that they do not have any preference.

2 | METHODS

2.1 | Fishes and husbandry

We used six populations in our study, three each for the two species involved (Table S1, Figure 1). Out of these three, one *P. mexicana* and one *P. latipinna* population each was from allopatry, the other two each from sympatry with *P. formosa*.

Generally, fishes were kept in large stock tanks at the *International Stock Center for Livebearing Fishes* (*ISCLF*) in a greenhouse located at the Aquatic Research Facility (ARF, 35.1832, −97.4477), which is maintained by the School of Biological Sciences of the University of Oklahoma in Norman. Stock tanks are population-specific and contain individuals of both sexes that are allowed to randomly outbreed. Tanks held either 500 or 1000 L, contained natural plants, and were connected to a flow-through system that is fed by well water. These large tanks contain a large amount of natural food that was supplemented with flake food (TetraMin) three times a week. Water temperature was maintained at approximately 28° but fluctuated somewhat with the temperature of the greenhouse. The light cycle was natural. Individuals from these stocks were moved into a dedicated fish room in preparation for experiments. There they were sorted into sex-specific 40 L tanks and maintained until experimentation was completed. After experimentation, the fishes were returned to their respective stock tanks for breeding. Consequently, nonvirgin *P. mexicana* and *P. latipinna* females originated from stock tanks where they were able to interact (and mate) with males from their own species and population. While it is highly likely that they did engage in prior matings, we cannot be sure of this. Also, their relatedness and familiarity with each other are not known. The F_1 individuals used here are offspring of two sibships with a *P. latipinna* mother and a *P. mexicana* father (note: this is opposite to the direction that led to natural Amazon mollies). At the time of the experiments, these were the only F_1s available.

Virgin (unmated) females were isolated in individual tanks at an age of about 30 days and subsequently raised individually in 9-L tanks either in dedicated racks in the greenhouse or in a fish room at a 12:12 h light cycle and fed daily ad libitum with flake food, supplemented with frozen *Artemia* nauplia, *Daphnia* and mosquito larvae. The temperature in this room was maintained at 28°.

2.2 | Choice tests

Using virgin and nonvirgin descendants of multiple populations of wild-caught *P. mexicana* and multiple populations of nonvirgin *P. latipinna*, including one wild-caught population (Airport Ditch), individuals of each species were tested in a typical choice experiment to examine the species preferences of the females of each species. Choice tests are considered to be a reasonable proxy for actual mating decisions (reviewed by Dougherty, 2020; Dougherty & Shuker, 2014; Witte, 2006).

In our experiments, females were placed in a clear Plexiglas cylinder in the center of a 40-l fish tank that was divided into thirds by lines drawn onto the tank (Figure 2).

This configuration has been used successfully many times (for recent examples, see Makowicz, Tanner, et al., 2016; Makowicz, Tiedemann, et al., 2016). For an experimental trial, a conspecific male and a heterospecific male were placed in their own cylinder; one in the left third of the tank and one in the right third. After 5 min of acclimating, the female was released from her cylinder and association time was recorded for a 5-min interval. The association zones were the left or right third of the tank, respectively (Figure 2). The central area of the tank served as neutral zone, where time was not counted. The female was then gently recaptured, and the cylinders of the males were moved to the opposite side, to allow detecting potential side biases. We considered it a side bias when a chooser fish spent more than 80% of its time on one side of the test tank, without discernible reason. We recorded the number of side biases, tested if they were associated with a particular side of the tank and whether the females of different species differed in the frequency of occurrence. Our procedure controls statistically for side biases. However, the number and direction of side biases can provide important information in itself, as high rates of side biases may be indicative of individuals not responding to the provided stimuli in a meaningful way and hence, we report the numbers and distributions of the side biases. A separate statistical analysis with the side-biased individuals resulted in similar statistical results (see Tables S1 and S2) The female fish were then given another 5-min acclimation period. After that, the female was again released, and association time was measured for another 5 min. Females were used only once in this experiment, but males had to be used more than once due to limited numbers of males of comparable size. Males were given a minimum of 24 h of rest before reusing them and we assume that reusing some males did not influence the results. Males were always matched for size. For population affiliation of these females, see Table S1. Figure 1 provides a map depicting the geographic origin of the populations used as well as example photos of *P. mexicana*, *P. latipinna* males, and an F_1 female.

Association time was measured using two stopwatches, one for each male. They were started every time the females mouth reached one of the thirds containing a male. After both halves of the trial were completed, the total time with each male was calculated and recorded. The position of the conspecific male was randomized before trials began using a random number generator that chose either a 1 or 2 with equal probability, meaning the conspecific was initially either on the left or right, respectively. The males in each trial were also measured for standard length (the tip of the snout to the caudal peduncle) so that they were within 1 mm of each other to remove the potential influence of male size. Additionally, 30% of the water in the tank was replaced before each acclimation period.

2.3 | F_1 females

Using 24 F_1 individuals, the same choice experiment was conducted with a few alterations. Females in this experiment were used twice, once with another F_1 individual (a male) and a *P. latipinna* male and again with another F_1 individual and a *P. mexicana*. The order of these two tests was assigned randomly. Trials using the same females were done with a minimum of 24 h of rest for the fish and males were never paired with the same F_1 female to reduce individual bias as well. The data used can be found in Table S2. All trials were video recorded using a Nikon digital camera; videos are available on request. With regards to STRANGEness, we believe we have obtained a sample that is as unbiased as possible, given the conditions outlined above.

2.4 | Statistical treatment

All statistical analyses were conducted in SPSS 29. All *p*-values are two-tailed. Specifically, we analyzed choice data with paired *t* tests and used univariate GLMs to explore the role of the species and population of the choosing individual in mate choice. The distribution of side biases was tested using a chi-square test.

3 | RESULTS

3.1 | The sexual ancestors, *Poecilia latipinna* and *Poecilia mexicana*

3.1.1 | Overall mate choice

We tested for an overall preference for conspecific partners using a paired *t*-test comparing association times (time with heterospecific vs. time with conspecific) (Figure 3). The mean time females spent with conspecific males was 205 (±131 SD) s; they spent slightly less time (167 (±115 SD) s) with heterospecific males. This is not statistically significantly different ($t = -1.68$, $n = 118$, $p = .095$). We compared the sizes of the stimulus males, too, but there was no significant difference ($t = -.89$, $n = 118$, $p = .37$). On average, conspecific males were 26.83 (±3.6) mm long, whereas heterospecific males

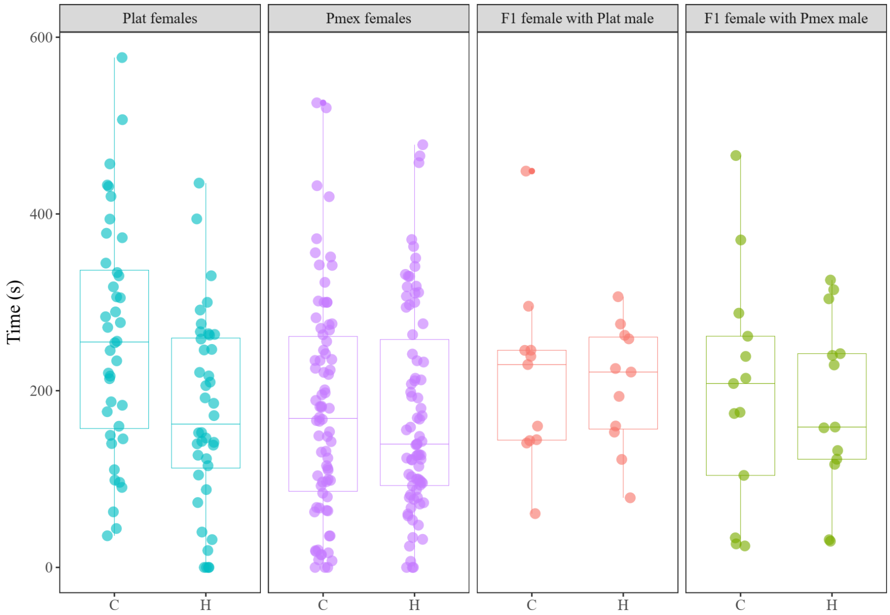

FIGURE 3 Boxplots of association times of the parental females, *Poecilia latipinna* (Plat), *Poecilia mexicana* (Pmex), and F_1 females with either a *P. mexicana* male or a *P. latipinna* male versus an F_1 hybrid male. C stands for conspecific and H for heterospecific. For F_1s, the conspecific was an F_1 male, and the heterospecific was a sexual male.

were 26.9 (±3.6) mm long. The females (or choosers) were on average 35.84 (±7.6) mm long.

Furthermore, we used a GLM comparing the two species, *P. mexicana* and *P. latipinna*, with species and population as factors and strength of preference (SOP), calculated as ((time with the heterospecific male + 1)/(time with heterospecific male + 1) + time with conspecific male), as the dependent variable. The size of the choosing female was used as a covariate. The resulting GLM found no significant effect of species (df = 1, $F = .508$, $p = .477$) or population (df = 5, $F = .677$, $p = .642$) on preference. There was no interaction and chooser size had no significant effect (df = 1, $F = 3.17$, $p = .077$).

3.1.2 | *Poecilia mexicana* from Tampico only

For Amazon mollies, the natural hybrid of a *P. mexicana* mother and a *P. latipinna* father, we have a fair estimate of the locality in which the hybridization possibly occurred ca. 100,000 generations ago, southwest of what is now Tampico, México (Costa & Schlupp, 2020) (Figure 1). We used females from a population that currently occurs in the general area which has been hypothesized to be historically syntopical and is known to be currently syntopical for the two parental species. We looked at this population separately using a paired *t*-test comparing the time in association with a *P. mexicana* and *P. latipinna* male. We were unable to detect a significant female preference of *P. mexicana* females for sympatric males ($t = .933$, $p = .36$, $n = 28$).

3.1.3 | Virgin (unmated) females

Furthermore, some of the females we used were unmated (virgin), while the majority of females had previously been with males which reflects the most likely natural state. Because we only had access to unmated *P. mexicana*, mating status was not included in the overall model but analyzed separately using a paired *t*-test comparing time with the conspecific (*P. mexicana*) versus the heterospecific (*P. latipinna*) male only for unmated females. There was no significant preference for conspecific males in unmated females ($t = -2.11$, $p = .834$, $n = 39$).

TABLE 1 Number of side biases to the left or right per species.

	Left	Right
Poecilia latipinna	9	3
Poecilia mexicana	10	17

3.2 | F_1 females

When given a choice between an F_1 male and a sexual male of the same size, F_1 females showed no preference ($t = -.293$, $p = .77$, $n = 24$).

3.3 | Side biases

Overall, we tested 118 individual females of *P. mexicana* and *P. latipinna* from different populations (see Table S1 for full data set). Many trials, 39 (33%), resulted in side biases (Table 1). We tested whether the directionality or the species of chooser differed from random using a chi-square test with Yates correction. Results indicated that the pattern did not significantly differ from random ($\chi^2 = 3.39$, $p = .65$).

In F_1 females out of the 24 trials, we detected seven side biases (21.16%), two of which were for the right side of the tank and five for the left side of the tank (Binomial test, $p = .4531$). Most F_1 females showed a side bias only with one combination of males, but one female showed a side bias in both combinations.

4 | DISCUSSION

4.1 | Overall mate choice and side biases

In our study, we attempted to mimic the hypothetical situation that must have led to the hybrid origin of the unisexual Amazon molly, *P. formosa*. Based on conclusive genetic evidence we know that Amazon mollies originated a single time from a mating of a *P. mexicana* female and a *P. latipinna* male (Warren et al., 2018). Our findings suggest that such a mating would not have been hindered by strong mating preferences for conspecific males. This is in general agreement with previous studies that found that *P. mexicana* females sometimes prefer males with a long-fin or *P. latipinna* phenotype (MacLaren & Rowland, 2006; Ptacek, 1998). Of course, this conclusion is only valid as much as mate preferences in *P. mexicana* have not changed much over the ca. 100,000 years of evolution that happened in the two sexual species since the formation of the hybrid (Warren et al., 2018). Side biases occurred in ca. 33% of the trials. Assuming nothing in our mate choice arena caused this high number of side biases, this supports our conclusion that there are no detectable preferences in our study, as females with a side bias may be unable or unwilling to discern between the stimuli offered. Furthermore, virgin females did not differ in their preferences from nonvirgin females, which supports findings from a meta-analysis that also found little effect on mating status across taxa (Richardson & Zuk, 2023). However, in other studies, nonvirgin and virgin females did differ in their preferences with nonvirgin Sailfin mollies showing a preference for larger males, whereas virgin females showed no significant preference (Ptacek & Travis, 1997).

We specifically tested individuals from a possible area of overlap, near Tampico, Mexico, but found no mating preference for conspecifics. We know from at least one published laboratory study and our own data that *P. mexicana* and *P. latipinna* from that area can be successfully crossed to produce sexual F_1s (Lampert et al., 2007; Miron Berbel-Filho et al., unpublished data). More generally, in our study, behavioral prezygotic isolation via female mate choice was not detected. One conclusion from this is that heterospecific matings are not selected against. Based on other studies, heterospecific matings are even predicted because of preexisting biases (MacLaren & Rowland, 2006; Ptacek, 1998). However, while this may help explain the historical origin of the Amazon molly, it makes it difficult to understand the apparent absence of natural F_1 hybrids of *P. mexicana* and *P. latipinna* in areas of overlap (Stöck et al., 2010).

Furthermore, we did not detect any population-based differences in female preferences. Such geographic differences in preference are known from other systems (e.g., in sticklebacks; Foster, 2013), poison frogs (Willink et al., 2013), as are geographic differences in behavior more generally (Foster & Endler, 1999; Gabor et al., 2013). Such differences are not universal, however. A study of male mate choice in *Limia perugiae*, a relative of mollies from the Caribbean, also found no differences in male association preferences over a large geographical area (Powell & Schlupp, 2024).

Given this and results from previous studies, how can we explain the absence of natural F_1 hybrids in the wild? One possibility is that isolating mechanisms are in place, but female mate choice is not one of them. Other prezygotic isolating mechanisms may also play a role, but other postmating prezygotic and/or postzygotic isolation may also be important. So far, we only know that F_1 individuals behaviorally differ from Amazon mollies (Dries, 2003; Makowicz & Travis, 2020), but a fitness comparison of F_1s with any of the parental species has not yet been done. F_1s do differ in some traits from parentals and have—for example—sex ratios that are female biased (Berbel-Filho & Schlupp, unpublished data; Lampert et al., 2007; Makowicz & Travis, 2020; Turner, Brett, Rasch, et al., 1980). Another possibility is that detecting hybrids in nature is generally difficult and unlikely (Contreras-Balderas, 1990; Janko et al., 2018). Genetic work may be useful to overcome this limitation and has shown widespread hybridization in livebearing fishes, for example, in the genus *Xiphophorus* (Cui et al., 2013), and in many other taxa (Moran et al., 2021).

There are caveats to our experiment, however, that need to be mentioned, also in the context of STRANGEness. The average size of the males and females used does not cover the natural size spectrum of the two species, especially the F_1 females were relatively small. Although, using small individuals may have led to weaker female preferences, choosing between two small males is clearly a natural situation for females. Furthermore, preferences of males can be influenced by the timing of the experiments relative to the hormonal cycle of the choosing females (Parzefall, 1973). Both male and female choice is more acute when females are in the short phase of their sexual cycle when their eggs can be fertilized. However, in many studies, females—often wild caught—with unknown mating status were used, and preferences are commonly detected (e.g., Ptacek & Travis, 1997). Our findings would be less surprising if mollies generally showed little or no preference for other mating-related traits, but this is not the case. In both species, *P. mexicana* and *P. latipinna* female (Schlupp, 2009) and male preferences (reviewed in Schlupp, 2018, 2021) for several traits have been documented. Males show preference for sexual females over Amazon mollies (Schlupp et al., 1994), *P. latipinna* females show a preference for larger mates (Gabor & Page, 2003; Ptacek & Travis, 1997; Witte & Ryan, 1998), and more symmetrical males (Schlüter et al., 1998). In *P. mexicana*, essentially the same pattern is found (Jordan et al., 2006; Plath et al., 2004; Sommer-Trembo et al., 2020; Zimmer et al., 2018). Together, these studies mirror more general findings in the family of Livebearing fishes, where on average females prefer more ornamented, larger males (Rios-Cardenas & Morris, 2011).

4.2 | F_1 females

The absence of a detectable preference in F_1 hybrids is not surprising given the lack of preference in parental species. We had predicted that the preference of the F_1s might have an inherited preference similar to that of the maternal ancestor, and this may indeed be the case. Our data would predict, however, that after the occurrence of sexual F_1s, indiscriminate mating may have led to a hybrid swarm. This was suggested by Alberici da Barbiano et al. (2013) as an early stage of the evolution of the Amazon molly and subsequently modeled by Fyon et al. (2023). This pathway, however, is not compatible with genomic data showing a single hybrid origin of the Amazon molly (Warren et al., 2018). Nonetheless, this single origin does not rule out the idea of complex interactions between parental species, F_1s, and beyond.

In the bigger picture, it seems important to evaluate the role of mate preferences in hybridization more generally. Clearly, in the case of the Amazon molly, some breakdown of isolation must have happened. We know this through the existence of Amazon mollies, but other such breakdowns may not leave behind such a large footprint (Janko et al., 2018, 2023). Indeed, while reports of actual hybrids between mollies in nature are rare, there is mounting evidence for a bigger role of hybridization than previously assumed (Moran et al., 2021; Schwenk et al., 2008). Furthermore, even in the presence of clear preferences, heterospecific matings may occur (e.g., Domínguez-Castanedo & Schlupp, 2023). This was argued in a study by McCoy et al. (2011), that investigated preferences for a novel trait, a mustache (Schlupp et al., 2010), that is only present in one of the species studied. Only that species, *Poecilia sphenops*, showed a *significant* preference for the mustache, but females from several other species showed *some* preference for the novel trait (McCoy et al., 2011).

Traditionally, hybridization is considered to be costly, and generally selected against, especially if the rate of overlap of the species is very limited and choice is rarely under selection. However, mounting evidence shows that hybridization is widespread and plays an important role in species evolution (Mallet, 2007). One excellent example is the origin of a new hybrid species in Galapagos Finches (Grant & Grant, 1992). In this case, the hybridization was directly observed, a situation that is highly unlikely, yet illustrates the crucial role of behavior in the origin of hybrids. Genetic studies are capable of providing evidence for such hybridizations (Lamichhaney et al., 2018) but cannot elucidate the behavioral processes that allow for hybridizations to happen. Our study may provide an example of the absence or a breakdown of prezygotic isolating mechanisms, allowing a hybrid species to appear and flourish.

AUTHOR CONTRIBUTIONS

Caden Smith: Investigation; writing – original draft; data curation. **Waldir Miron Berbel-Filho:** Conceptualization; investigation; writing – review and editing; methodology; visualization. **Montrai Spikes:** Methodology; writing – review and editing. **Frederic Fyon:** Conceptualization; writing – review and editing. **Francisco Úbeda:** Conceptualization; writing – review and editing; supervision; funding acquisition. **Ingo Schlupp:** Conceptualization; funding acquisition; writing – review and editing; methodology; visualization; formal analysis; project administration; data curation; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data analyzed for this study are available in the supplementary material. Video recordings of the trials are available on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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