Discriminating males alter sperm production between species

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Prezygotic reproductive isolation and its importance in speciation is traditionally approached from the viewpoint of those events that occur before mating. However, recent interest in sperm competition theory has shown that prezygotic isolation can be affected by mechanisms that occur after mating but before fertilization. One neglected aspect of these studies is how the cost of sperm production might play a role in species isolation. We examined differential sperm production in a species whose males are sexually parasitized by a unisexual gynogenetic species. Gynogens are clonal females that require sperm from males of closely related bisexual species to initiate embryogenesis. We tested for differential sperm production by male sailfin mollies (*Poecilia latipinna*) when they were in the presence of either a heterospecific, gynogenetic female (*Poecilia formosa*, Amazon molly) or a conspecific female. We found that previously demonstrated male mate choice for conspecific over heterospecific females also is revealed in sperm production. Males from both an allopatric and a sympatric population produce more sperm when in the presence of a conspecific female than when in the presence of a heterospecific female. We suggest that differential sperm production also could play a role in prezygotic reproductive isolation in bisexual species complexes that occur in sympatry.

Poecilia | prezygotic reproductive isolation | sperm competition | unisexual–bisexual species complex

Reproductive isolation between closely related species is thought to be one of the most important stages in the process of speciation (1). Prezygotic reproductive isolation and its importance in speciation is traditionally approached from the viewpoint of those events that occur before mating, such as assortative mating, or mate choice. Empirical evidence suggests that traits that are used in mate choice within a species may also be involved in maintaining sexual isolation between closely related species (2–5). Recently, the emphasis in studies of reproductive isolation has shifted to acknowledge the importance of events that affect fertilization success or failure that occur after copulation, but before zygote formation (6). For example, in a recent review, Howard (1) demonstrated the importance of conspecific sperm precedence in limiting gene exchange and suggested that such barriers may be quite ubiquitous in both plants and animals. Here, we propose that variation in sperm production could be a mechanism of isolation that is affected by precopulatory events such as mate choice, and that it may subsequently affect postcopulatory factors such as conspecific sperm precedence.

Many of the studies that examine the link between sexual selection and sexual isolation between species focus on systems in which the species of interest are bisexual and reproduce by means of "normal" sexual recombination (reviewed in refs. 4 and 5), and very few studies have addressed the importance of male mate choice or male behaviors in promoting sexual isolation (for exceptions, see refs. 7–10). One type of system in which it is particularly interesting to study male choice and sexual isolation is species assemblages that consist of both bisexual as well as unisexual, gynogenetic species. Gynogenetic species consist of only females but require sperm from males of closely related bisexual species to initiate embryogenesis (reviewed in refs. 11 and 12). Males that mate with these parasitic females gain no offspring and thus do not directly increase their fitness. Therefore, we expect strong selection on males to avoid heterospecific matings.

Although there are studies on how male choice can be revealed in sperm allocation (13, 14), few studies have examined how male mate choice might be revealed in sperm production (15), and none that we are aware of examine how sperm production might play a role in reproductive isolation. Spermatogenesis can be energetically costly and limit male reproductive success (13, 16–18), although the exact physiological mechanisms of such costs are poorly understood (13). In some species, when males are provided with stimuli from females, they exhibit an increase in sperm production (15, 19). These physiological changes are referred to as the priming response (20) and might represent mechanisms by which males can conserve energy associated with sperm production (21). Aspbury and Gabor (15) found that male sailfin mollies (*Poecilia latipinna*) in the presence of conspecific females for 7 days produced more sperm than those that were not presented with a female. The availability of females therefore has a clear effect on how much sperm males have ready to transfer to females. The existence of such a response, which may have evolved under selection pressures to conserve energy (19), may have been coopted as an adaptation for males to exercise male mate choice. Aspbury and Gabor (15) found partial support for this hypothesis in sailfin mollies by demonstrating that an association preference for larger females by male sailfin mollies (22, 23) also is associated with a greater sperm-priming response; i.e., males produce more sperm when presented with larger females (15).

The sailfin molly provides a model system for examining the potential effects of male choice on sperm production. Male sailfin mollies are sexually parasitized by the Amazon molly, *Poecilia formosa*, which is an all-female clonal species of hybrid origin that reproduce by gynogenesis (11, 12, 24). They must coexist and mate with males of the parental species (*P. latipinna* and *Poecilia mexicana*) to induce embryogenesis, but inheritance is strictly maternal (11, 12, 25). An unresolved question is why male sailfin mollies mate with Amazon mollies. The maintenance of Amazon mollies should be evolutionarily unstable because natural selection should work against males that mate with another species. One proposed explanation for the maintenance of Amazon mollies follows from heterospecific mate-choice copying. Mate-choice copying occurs when one female chooses to mate with a particular male after observing another female mate with that same male (26). Schlupp *et al*. (27) found that female sailfin mollies copy the mate choice of Amazon mollies, which implies that heterospecific mate-choice copying by female sailfin mollies could increase a male sailfin molly's future reproductive success.

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Abbreviation: SL, standard length.

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Despite the potential benefit of mate-choice copying, previous research indicates that there are significant costs to males that mate with Amazon mollies. In particular, male sailfin mollies show a greater mating preference for conspecifics than for Amazon mollies, and males from sympatric populations have a greater preference for conspecifics over Amazon mollies than males from allopatric populations (28, 29). These results suggest that mating with Amazon mollies is costly, as observed by both the general avoidance of Amazon mollies as well as the across-population variation in male mating preferences. In addition to the lack of direct fitness benefits through mating with Amazon mollies, there are likely energetic costs of time spent mating, sperm production, and sperm transfer. One potential mechanism for male sailfin mollies to reduce sperm production costs when in the presence of Amazon mollies would be to produce less sperm. Furthermore, we might expect this decrease in sperm production to be more obvious in male sailfin mollies from populations that are sympatric with Amazon mollies than in males that are from populations allopatric with Amazon mollies. This prediction follows from the observation of reproductive character displacement in male sailfin molly mate preferences for conspecifics over heterospecifics (29). Reproductive character displacement [greater degree of divergence in a reproductive isolating trait between closely related species in areas of sympatry relative to in allopatry (30)] can occur if receivers of information conveyed by traits indicating mate quality diverge in their mating preferences (28, 29, 31, 32), which could be revealed by differential sperm production.

Our objective in this study is to test for differential sperm production by male sailfin mollies when they are in the presence of either a heterospecific (Amazon molly) female or a conspecific female. We expect that the previously demonstrated male mate choice for conspecific over heterospecific females (28, 29) is revealed also in sperm production. We test this hypothesis using male sailfin mollies from both a sympatric and an allopatric population. Our results are discussed in light of their potential importance in reproductive isolation.

Methods

These experiments were conducted in the summers of 2003 and 2004. The fishes used for the sympatric treatment (both sailfin and Amazon mollies) in these experiments were collected March 2003 from a population in Tamaulipas, Mexico [universal transverse mercator (UTM) coordinates: 25.07, −98.02]. The male and female sailfin mollies used for the allopatric treatment were collected from a population in San Marcos, TX (UTM coordinates: 29.89, −97.93), and the Amazon mollies used for the allopatric treatment were collected from a population near Martindale, TX (UTM coordinates: 29.85, −97.84). Females and males were housed separately in 38-liter tanks for at least 30 days before the experiments. Because female *Poecilia* spp. have a 30-day ovarian cycle, this separation allowed females to have dropped any broods they may have held before testing. Fish were fed O.S.I. Spirulina Flake mixed with O.S.I. Freshwater Flake food (Ocean Start International Marine Laboratory, Hayward, CA) and supplemented with live brine shrimp and maintained on a 14-h:10-h light:dark cycle.

The following methods were the same for both the sympatric and the allopatric populations. On day 0 of the experiment, males were removed from their stock tank and anesthetized in 400 ml of water containing several drops of clove oil. The following sperm extraction and counting methods have been successfully used previously in sailfin mollies (15). After measuring male standard length (SL), males were placed along the edge of a shallow Petri dish lined with wet cotton with their ventral side up. Gentle pressure was applied to the side of the male, going from behind the eye laterally to the base of the anal fin. Spermatozeugmata (sperm bundles) came out at the base of the gonopodium and were collected by using an aspirator [an aspirating mouthpiece attached to airline tubing, connected to a small (5-cm) glass tube, with a 1- to 200-μl gel-loading pipette tip attached at the end]. This process was repeated on each male until no more spermatozeugmata were expelled from the male. The spermatozeugmata were placed into a microcentrifuge tube with 100 μl of 0.9% saline solution (0.9 g of NaCl per 100 ml of water), and repeatedly drawn up and expelled from a pipette (to distribute sperm cells evenly). Sperm cells were counted five times on an improved Neubauer chamber hemocytometer (Reichert, Buffalo, NY) under ×400 magnification. The total number of sperm cells was determined by multiplying the mean cell count by the sample's initial volume (100 μl) and dividing by the volume of the hemocytometer (0.1 μl). Sperm counts were made blind to the treatment identity of the males.

After sperm extraction on day 0, individual males were placed in separate 18-liter tanks, each divided into two sections by a clear Plexiglas divider. The dividers were not sealed to the tank, allowing access to both visual and chemical cues. For both the sympatric and the allopatric populations, half of the males were randomly assigned to the conspecific female treatment and the other half to the heterospecific female treatment. On day 3 of the experiment, the stimulus female (either an Amazon or a sailfin molly) was placed in the other section of the tank. [In a pilot study, we found that 3 days is sufficient time for males to recover to "baseline" sperm counts by comparing sperm stripped from males on day 0 vs. day 3 when males were held in physical, but not visual, isolation from conspecifics (paired t test, df = 18, t = 0.052, P = 0.959).] On day 10, all fish were removed from the test tanks, and the number of sperm available from each male was counted again. The priming response was defined as the difference: (day 10 sperm counts) − (day 0 sperm counts).

Because we were limited by the available number of males and females from our populations, we could not control for the size ranges of all test fishes. Therefore, we first examined whether there were differences in male and/or female SL between the treatments. Furthermore, we examined whether there were differences in initial sperm counts (day 0) between males from the two populations. These analyses are necessary, because larger male sailfin mollies produce more sperm, and males show a heightened priming response when with larger females (conspecific) as compared with smaller females (15).

Within each population, we compared the priming response between males in the conspecific and heterospecific treatments. We also compared the difference in the priming between the two populations within the female species treatments. Based on Bonferroni's inequality (33), we reduced α because each priming data set was analyzed twice ($\alpha = 0.05/2$). We also tested whether the amount of sperm stripped from males on day 10 was affected by the female treatment. However, because of correlation between day 0 and day 10 counts, we analyzed the residuals of day 10 counts from a linear regression with day 0 counts as the independent variable. All tests are two-tailed. Before analyses, we tested the assumptions of parametric tests (such as unpaired t tests) using the Kolmogorov–Smirnov test for normality and the F test for homogeneity of variances (33).

Results

There was no difference in male SL between the treatments both in sympatry and in allopatry. [In sympatry, mean male SL (±SEM) was as follows: heterospecific, 30.59 ± 1.67; conspecific, 31.54 ± 1.42; unpaired t test, df = 25, t = 0.436, P = 0.666. In allopatry, mean male SL (±SEM) was as follows: heterospecific, 32.41 ± 1.94; conspecific, 30.31 ± 1.54; unpaired t test, df = 26, t = 0.848, P = 0.404.] Likewise, there was no difference in female SL between the two female species both in sympatry and in allopatry. [In sympatry, mean female SL ± SEM was as follows: heterospecific, 36.89 ± 0.27; conspecific, 36.75 ± 0.36;

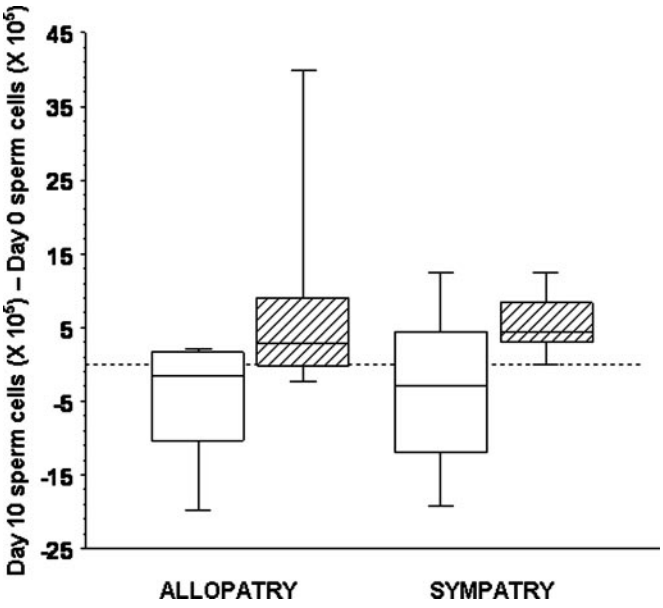

Fig. 1. Box plots representing male sperm-priming response (day 10 − day 0 total sperm cells) for males from one allopatric population (ALLOPATRY) and one sympatric population (SYMPATRY). The open boxes represent males in the heterospecific female treatment, whereas the hatched boxes represent males in the conspecific female treatment. Data above the dashed horizontal line at $y = 0$ indicate an increase in sperm production from the initial sperm count, whereas data below the line indicate a decrease in sperm production. The upper and lower horizontal lines of the boxes represent the first and third quartiles, the middle horizontal lines represent the medians, and the bars indicate the range (33).

unpaired t test, df = 25, $t = 0.315$, $P = 0.755$. In allopatry, mean female SL ± SEM was as follows: heterospecific, 35.87 ± 0.79; conspecific, 38.54 ± 1.2; unpaired t test, df = 26, $t = 1.845$, $P = 0.077$.]

Males from the two populations did not differ in their day 0 sperm counts. [Mean day 0 sperm counts ± SEM were as follows: sympatry, $12.35 \times 10^5 \pm 2.01 \times 10^5$; allopatry, $15.90 \times 10^5 \pm 3.50 \times 10^5$; unpaired t test, df = 53, $t = 0.869$, $P = 0.389$.]

In both the allopatric population and the sympatric population, males primed significantly more sperm when in the presence of a conspecific female for 1 week than did males in the presence of a heterospecific female (Fig. 1; sympatry: Mann–Whitney U test, $N_{\text{conspecific}} = 14$, $N_{\text{heterospecific}} = 13$, $z = -2.378$, $P = 0.017$; allopatry: Mann–Whitney U test, $N_{\text{conspecific}} = 14$, $N_{\text{heterospecific}} = 14$, $z = -2.688$, $P = 0.007$). We also explored the effect of female species on the day 10 total sperm counts in each population by using a Mann–Whitney U test on the residuals of day 10 sperm counts. As with the priming response, we found that day 10 counts were significantly greater for males in the conspecific female treatment than in the heterospecific treatment (sympatry: Mann–Whitney U test, $N_{\text{conspecific}} = 14$, $N_{\text{heterospecific}} = 13$, $z = -2.426$, $P = 0.015$; allopatry: Mann–Whitney U test, $N_{\text{conspecific}} = 14$, $N_{\text{heterospecific}} = 14$, $z = -2.619$, $P = 0.009$).

There was no difference in the priming response for either conspecific or heterospecific female treatments between males from the sympatric population and those from the allopatric population (Fig. 1; conspecific: Mann–Whitney U test, $N_{\text{sympatry}} = 14$, $N_{\text{allopatry}} = 14$, $z = -0.988$, $P = 0.323$; heterospecific: Mann–Whitney U test, $N_{\text{sympatry}} = 13$, $N_{\text{allopatry}} = 14$, $z = -0.291$, $P = 0.771$).

Discussion

We found that male sailfin mollies from two different populations in the presence of conspecific females for 7 days primed more sperm than those that were presented with a heterospecific female. Male sailfin mollies can discriminate between conspecific and heterospecific females and adjust their potential reproductive investment accordingly. This result is consistent with the demonstration of male sailfin molly mating preferences for conspecific over heterospecific females (28, 29) and suggests that, even though males may experience selection to mate with Amazon mollies in situations where heterospecific mate-choice copying occurs, they could minimize the costs of these matings by transferring less sperm. This result, along with previous work on the effect of mate quality on sperm priming (15), supports the hypothesis that sperm production is a trait that both may play an important role in sexual selection as well as serve as a prezygotic reproductive barrier.

In this study, we were interested in determining whether the previously demonstrated reproductive character displacement in male mating preferences for conspecifics over heterospecifics (28, 29) was also revealed in sperm production. We did not detect a significant difference in the priming response between our allopatric population and our sympatric population. There are several potential reasons why we did not find a significant difference. First, as pointed out by Gabor and Ryan (29), there could be substantial variation within sympatry and allopatry such that our selection of just one population in each could obfuscate any real differences between allopatric and sympatric populations. This is a hypothesis that needs further exploration. Another hypothesis that could explain the lack of a difference in sperm priming between the allopatric and sympatric population is that our allopatric population is an introduced population [from Louisiana and Florida after 1941 (29)]. However, this seems like an unlikely explanation because the source populations also are allopatric and had no evolutionary history with Amazon mollies. Finally, our lack of a difference in sperm priming between allopatry and sympatry could be a biologically meaningful result, indicating that perhaps, although males in allopatry and males in sympatry differ in their degree of mating preference for conspecifics, this difference in mating preferences does not correlate with differences in sperm priming.

The pattern of greater sperm priming for conspecific females in both allopatry and sympatry is similar to the results on male mating preferences for conspecific females in both allopatry and sympatry (28, 29). Ryan *et al*. (28) proposed that the preference for conspecific females is likely not the result of direct selection against males that mate with Amazon mollies, but is rather a consequence of general male preferences for conspecifics. Our results, along with previous studies, support this hypothesis. Male sailfin mollies prefer to associate with larger females (22, 23) and show a heightened priming response for larger females (15). In addition, females that have broods from multiple sires have higher fecundity than average for their body size (34). Therefore, the general increase in sperm production for conspecific females over heterospecific females may reflect a continuation of the mate-quality preferences of these male sailfin mollies, providing evidence that the same traits that are selected by sexual selection can affect interactions between closely related species, and potentially lead to reproductive isolation (2, 4, 5).

Conspecific sperm precedence has been recognized as an important aspect of prezygotic reproductive isolation (1). In many species, when a female is mated sequentially to a conspecific and a heterospecific male, most progeny are sired by the conspecific male, regardless of the mating order (35–39). In the closely related sympatric species of ground crickets (*Allonembius fasciatus* and *Allonembius socius*), there is a very low level of hybrids found naturally (1, 40), suggesting strong reproductive isolation. Through a series of studies, Howard and colleagues (1, 41) have shown that isolation is not due to premating factors such as mate choice by either sex or ecological differences between

the species. In addition, there is little evidence of postzygotic barriers (1, 42). These studies certainly suggest that conspecific sperm precedence plays a role in the reproductive isolation of the two species. What we suggest here is that conspecific sperm precedence might have a link to differential sperm production when males are in the presence of heterospecifics.

Although the emphasis of our study was not on explaining what were the relative benefits and costs for male sailfin mollies of mating with heterospecifics, our results may shed light on how males might lower the physiological costs associated with sperm production. If it indeed does benefit male sailfin mollies to mate with Amazon mollies as a result of heterospecific mate choice copying (27), these males could reduce costs associated with the mating by producing and ultimately transferring less sperm to Amazon mollies. We are currently examining how sperm priming relates to sperm transfer in this system. Results from guppies (*Poecilia reticulata*) are inconsistent on the relationship between volume of sperm stripped and volume of sperm actually ejaculated in mating or male fertilization success (43, 44). One study demonstrated that there is a positive relationship between the amount of sperm stripped and the amount transferred to females (43). However, in another study (44), sperm stripped from males was not a good predictor of fertilization success, although the amount of sperm actually delivered at copulation was not measured. These studies suggest that, to understand the functional significance of measuring stripped sperm, we also must examine whether males can adjust sperm transfer facultatively (44). In our current studies on the effect of female species, the lack of a relationship between sperm priming and sperm stripped could have multiple interpretations. Specifically, males are likely under selection pressures resulting from interactions with conspecific females to tailor ejaculate sizes in response to variation in female size and variation in perceived risk of sperm competition. Such selection may ultimately constrain male abilities to tailor ejaculate expenditure when in the presence of a heterospecific that is of hybrid origin. If the sperm production decreases observed in sailfin mollies when in the presence of heterospecific females are mirrored by actual sperm transfer, then we would have stronger support for the hypothesis that differential sperm production between heterospecific and conspecific females can play a role as a prezygotic reproductive barrier and play a role in establishing conspecific sperm precedence.

In conclusion, we demonstrate that males' discrimination abilities between heterospecifics and conspecifics are revealed in sperm production. We suggest that the role of sperm production in reproductive isolation should be examined in hybrid zones between bisexual species.

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