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ORIGINAL PAPER

Differential sperm expenditure by male sailfin mollies, *Poecilia latipinna*, in a unisexual–bisexual species complex and the influence of spermiation during mating

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Abstract Selection should favor strategies that reduce costs associated with spermatogenesis. This is especially true when males are sympatric with closely related species, and must avoid heterospecific matings, as in the unisexual–bisexual species complex of mollies. Male sailfin mollies, *Poecilia latipinna*, are sexually parasitized by Amazon mollies (*P. formosa*), and produce more sperm in the presence of female sailfin mollies than in the presence of Amazon mollies. We tested the hypothesis that male sailfin mollies differentially expend sperm when mating with either conspecific or heterospecific females. We measured sperm expenditure by determining the amount of sperm males have remaining after mating. Male sailfin mollies had more sperm available after mating with female sailfin mollies than after mating with Amazon mollies. While this result could indicate higher sperm expenditure to Amazon mollies, males mating with female sailfin mollies had more sperm available after mating than their baseline sperm reserves. Spermiation, the last stage of spermatogenesis, could be triggered by physical contact with females, and could increase sperm availability during mating. We examined the relationship between sperm availability and the amount of time that males mated with females. We found that sperm availability increased as mating trial time increased with female sailfin mollies, but not with Amazon mollies. Spermiation in the presence of conspecific female stimuli could reduce physiological costs associated with spermatogenesis while increasing the amount and quality of sperm available for sperm competition. We suggest that future studies examining sperm priming and expenditure should consider the potential for spermiation.

Keywords *Poecilia* · Spermiation · Male mate choice

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Introduction

It is increasingly evident that sperm production is costly to males (Dewsbury 1982; Nakatsuru and Kramer 1982; Shapiro et al. 1994; reviewed in Wedell et al. 2002; Aspbury and Gabor 2004a, b). One expected outcome of costly sperm production is differential control of sperm production and expenditure. Increased sperm production in the presence of desirable females may increase male mating success and can indicate male mating preferences (Aspbury and Gabor 2004a, b). Recent studies suggest that, in some species, males may increase sperm allocation to females exhibiting traits correlated with higher fecundity (e.g., fish, *Thalassoma bifasciatum*, Shapiro et al. 1994; *Salvelinus alpinus*, Masvaer et al. 2004; birds, *Gallus gallus*, Pizzari et al. 2003; crayfish, *Austropotamobius italicus*, Rubolini et al. 2006). Facultative adjustment of sperm expenditure may allow males to budget energy associated with sperm production.

One scenario where control of sperm production and sperm expenditure should be strongly favored is within a species assemblage that consists of a bisexual species and a closely related unisexual gynogenetic species (Gabor and Ryan 2001; Aspbury and Gabor 2004b). Gynogenetic species consist of only females but require sperm from males of closely related bisexual species to initiate embryogenesis (Hubbs and Hubbs

1932; Balsano et al. 1989). The Amazon molly, *Poecilia formosa*, is a gynogenetic species of hybrid origin that requires sperm from males of either parental species, sailfin mollies, *P. latipinna*, or Atlantic mollies, *P. mexicana* to initiate embryogenesis (Hubbs and Hubbs 1932; Kallman 1962; Darnell et al. 1967). The Amazon molly is a livebearing fish native to rivers and streams along the Gulf Coast from Veracruz, Mexico to southern Texas, USA. This clonal, all female, species formed from a single hybridization event that occurred as far as 100,000 years ago, based on genetic evidence (Avise et al. 1991; Schartl et al. 1995; but see Dries 2000, 2003). The sailfin molly is a livebearing fish native to brackish waters of southern Mexico and the southern USA near Rio Tuxpan along the Gulf of Mexico through North Carolina. Throughout the southern part of the sailfin molly range, they are sympatric with Amazon mollies. Populations of sailfin mollies in the northern and eastern extent of their range are allopatric from Amazon mollies.

Male sailfin mollies cannot directly increase their fitness by mating with Amazon mollies because the male genes are not incorporated into offspring, although males may indirectly increase their fitness when female sailfin mollies copy the mate choice of Amazon mollies (Schlupp et al. 1994). Male sailfin mollies prefer to mate with female sailfin mollies over Amazon mollies if given a choice, and more so in populations sympatric with Amazon mollies (Hubbs 1964; Ryan et al. 1996; Gabor and Ryan 2001). Males also show a stronger association preference for female sailfin mollies over Amazon mollies (Schlupp et al. 1994; Gumm et al. 2006). Male sailfin mollies produce more sperm when in the presence of female sailfin mollies than when in the presence of Amazon mollies when provided with both chemical and visual cues (Aspbury and Gabor 2004b). When male Atlantic mollies, *P. mexicana* (the other parental species of Amazon mollies) mated with female Atlantic mollies and Amazon mollies in a choice experiment, more sperm was recovered from the reproductive tract of female Atlantic mollies than from Amazon mollies (Schlupp and Plath 2005). In our study, we examined the hypothesis that male sailfin mollies expend more sperm when mating with conspecific females. One prediction of this hypothesis is that males will have less available sperm after mating with a conspecific female than after mating with a heterospecific Amazon molly.

One process that could make interpretations of sperm expenditure data difficult is spermiation. Spermiation is the last stage of spermatogenesis in which spermatids undergo final maturation and are released from the Sertoli cells into the efferent duct system (Grier 1973; Grier et al. 1980). In some fish species, the presence of sexually mature conspecific females stimulates an increase in plasma gonadotropin (GtH) and an increase in "ready" (*sensu* Bozynski and Liley 2003) sperm for ejaculation (Stacey and Sorensen 1991; Liley et al. 1993; Olsén and Liley 1993). In addition, spermiation can be induced with injections of GtH in the absence of female stimuli (Billard et al. 1990). During mating in a species with year-round spermiation (as is found in poeciliid fishes—Billard et al. 1990), the rate of spermiation may increase beyond the rate of sperm expenditure, which could lead to males having more sperm available after mating, relative to their "resting", or baseline measure of sperm availability. If spermiation occurs continuously at a rate that exceeds sperm expenditure, the increase in sperm availability would be more pronounced as the length of the mating trial increases. We therefore performed a second experiment to examine the potential for spermiation during mating in male sailfin mollies by determining how sperm availability is affected by the amount of time a male can interact with either a conspecific female or a heterospecific Amazon molly.

Materials and methods

All fish were maintained at Texas State University in 38-l (54×29×33 cm) and 53-l aquaria (76×32×32 cm). Fish were maintained on a 14-h light/10-h dark cycle using UV and fluorescent lighting to simulate daylight (40 W Coralife Day-Max Aquarium daylight, 40 W Coralife Actinic 03 Blue, 40 W Coralife 10,000 k high-intensity purified super daylight, and 40 W General Electrics fluorescent), and fed Ocean Star International Spirulina Flake mixed with Ocean Star International Freshwater Flake food twice daily until satiation and supplemented daily with live brine shrimp. Females were isolated for a minimum of 30 days in single sex tanks. Only non-postpartum females were used. Males were isolated for at least 7 days in single sex tanks.

Experiment 1: patterns of sperm availability between species

We used male and female sailfin mollies originating from a population sympatric with Amazon mollies in Tamaulipas, Mexico (field collected in 2003), and Amazon mollies originating from another population sympatric with sailfin mollies also in Tamaulipas, Mexico (field collected in 1989 and 1998).

Mating trials were conducted from August–October 2004 between 0800 hours and 1700 hours. On day zero of the experiment, male SL (N=24, mean SL±SEM=30.0±1.8 mm, range=21.1–49.7 mm) was recorded and sperm was extracted following established protocols (Aspbury and Gabor 2004a, b). Removing sperm on day 0 resulted in males only having sperm available that was produced in the presence of the stimulus females. Following sperm extraction, males were placed in separate 18 l aquaria. One female conspecific and one female heterospecific (size matched± 2 mm SL; mean SL±SEM sailfin molly=35.2±1.0 mm,

range=32.0–51.0 mm, N=24; mean SL±SEM Amazon molly=35.8±1.1 mm, range=32.2–52.5 mm, N=24) were haphazardly paired with each male. Aquaria were divided in half with a clear Plexiglas divider with the male on one side and both females on the other, providing visual cues from both species of female to the male. The Plexiglas divider was not sealed to the aquaria, providing chemical cues from both species of females to the male.

We began the first mating trial on day 3 of the experiment because previous research indicates that 3 days is a sufficient amount of time for males to rebuild sperm stores (Aspbury and Gabor 2004a). One female, picked randomly, was removed from the tank. After removing the tank divider the male was allowed to physically interact with the remaining female for 60 min; timing started with the first mating attempt (gonopodial thrust). During the first 10 min of the mating trial, the number of gonopodial thrusts directed at the female was recorded. If the male did not attempt to mate with the female within 60 min, the trial ended. Most observed mating attempts occurred during the first 5 min of the trial. Sperm was extracted from the male immediately after the mating trial. After sperm extraction, all fish were returned to the test tank for another 3 days for the male to replenish sperm stores before the mating trial with the second female.

The mating trial was repeated on day 6 with the female that was not mated in the first trial. After completion of the second mating trial, all fish were returned to the test tank for another 3 days for the male to rebuild sperm stores. On day 9, we extracted sperm from the male for a measure of sperm produced in the presence of the stimulus females, without physical contact. This was our measure of the male's baseline sperm amount when in a mixed-species social environment. Baseline measurements of sperm availability increase with both male size and the size of stimulus females (Aspbury and Gabor 2004a, b; this study: day 9 sperm count=$-4.0\times10^7+6.1\times10^5$ (average female SL)$+8.0\times10^5$ (male SL); $F_{2,21}$=24.40, P<0.0001, r^2=0.69). Because we were interested in examining sperm availability after mating relative to the male's baseline amount of sperm, we subtracted the sperm stripped from males on day 9 from the sperm remaining in males after each mating trial. Positive values were possible when the amount of sperm remaining after mating was higher than the amount available when males did not have physical access to females (baseline). Sperm samples were coded so that species identity of the female mated during the trial could not be identified when counting. Mating trials were included for analysis if males attempted to mate with one or both females (N=24).

Control experiment

To determine if male sperm production decreases across days, irrespective of the presence of mating stimuli, we tested males (N=10) in the same conditions as outlined above in May 2005. In these tanks, sperm was stripped from each male on the same days as the males in the experimental groups, but these males never participated in mating trials. We compared the amount of sperm stripped from males across the four sperm samples (days 0, 3, 6, and 9) using repeated measures ANOVA.

Experiment 2: patterns of sperm availability over time

Sailfin mollies were collected from Spring Lake, Texas (the headwaters of the San Marcos River). Amazon mollies used in the following experiment originated from another population sympatric with sailfin mollies from the San Marcos River near Martindale, Texas. Mating trials were conducted from March to June 2006 and started between 0830 hours to 1030 hours. On day 0 of the experiment, we extracted sperm from the males so that the only sperm available for mating was produced during the trial. After sperm extraction, one female was placed opposite one male in an 18-l aquarium divided in half with a clear Plexiglas divider, providing visual and chemical cues to the male. Male sailfin mollies (mean±SEM SL: 29.0±0.7 mm, range=20.6–46.8 mm, N=160) were placed with either a conspecific female (mean±SEM SL: 39.5±0.7 mm, range=28.8–58.2 mm, N=80), or an Amazon molly (mean±SEM SL: 42.2±0.4 mm, range=34.9–49.9 mm, N=80) and these pairs were assigned to one of five treatments (N=16 per treatment) differing in the amount of time provided for mating: (1) 10 min; (2) 60 min; (3) 120 min; (4) 240 min; and (5) 480 min. Males were not used for more than one trial. Neither male SL nor female SL differed among treatments when males were paired with conspecific females (male SL, ANOVA: $F_{4,75}$=0.417, P=0.800, conspecific female SL, ANOVA: $F_{4,75}$=2.078, P=0.092) nor when they were paired with a heterospecific female (male SL, ANOVA: $F_{4,75}$=0.350, P=0.844, Amazon SL, ANOVA: $F_{4,75}$=0.644, P=0.633).

On day 3 the divider was removed and the male could physically interact and potentially mate with the female for 10–480 min depending on the treatment; trial time started with the first mating attempt. Immediately after trials, sperm was extracted from the male. The mated male and female were once again returned to the divided tank for another 3 days for the male to rebuild sperm stores. On day 6 sperm was extracted from the male to obtain the baseline amount of sperm produced in the presence of a female, but without physical contact, and male and female SL were measured. To determine sperm availability after mating relative to the male's baseline amount of sperm, we subtracted the sperm stripped from males on day 6 from the sperm remaining in males on day 3. As with experiment 1, positive numbers indicate that more sperm was available after mating than the amount of sperm available without mating. Sperm samples were coded

so that female species and trial time could not be identified when counting.

Statistical analyses

Experiment 1: patterns of sperm availability between species

We used a repeated measures analysis to test how sperm availability was affected by the treatment (female species; fixed effect), mating order (fixed effect), whether males mated with the Amazon molly (fixed effect), the covariate of mating attempts, and the repeated effect of male identity (random effect). Including male identity as a factor controls for effects due to individual males, including variation in SL. Because females were size matched, female SL was not included in the analysis. For the control experiment we used repeated measures ANOVA to examine if sperm availability in the absence of physical contact with females decreased or increased across days. These analyses were performed using JMP 6.0 (SAS Institute). Alpha was set at 0.05 and all tests were two-tailed.

Experiment 2: patterns of sperm availability over time

We used ANOVA to determine whether male SL and female SL differed among treatments when males were paired with either a conspecific female or an Amazon molly. A cubed root transformation was used on the sperm availability data to meet the assumptions of parametric tests while maintaining the relationship among the data for both groups of males. We used simple linear regressions to determine the relationship between sperm availability and mating trial time for each data set. These analyses were performed using JMP 6.0 (SAS Institute), and alpha was set at 0.05.

Results

Experiment 1: patterns of sperm availability between species

Male sailfin mollies were more likely to direct mating attempts at sailfin mollies than Amazon mollies (Fisher's exact test on gonopodial thrusts: P=0.002, N=24/24 males attempting to mate with the sailfin molly and N=15/24 males attempting mating with the Amazon molly). Therefore, we included whether males directed mating attempts toward the Amazon molly as a factor in the analysis. Sperm availability was not affected by whether males directed mating attempts at Amazon mollies (ANOVA: $F_{1,19}$=2.691, P=0.117). There was no significant effect of mating order (ANOVA: $F_{1,19}$=0.262; P=0.615) or of the number of mating attempts (ANOVA: $F_{1,19}$=0.038; P=0.261) on sperm availability. There was no significant interaction between female species and mating attempts ($F_{1,20}$=1.344; P=0.261). There was a significant effect of female species (ANOVA: $F_{1,20}$=6.883; P=0.017). Males had more sperm available after mating with sailfin mollies than after mating with Amazon mollies (Fig. 1).

In the control experiment, we found no significant increase or decrease in sperm stripped from non-mated males during the full length of the experiment (ANOVA: $F_{3,27}$=0.577, P=0.635).

Experiment 2: patterns of sperm availability over time

There was a significant positive relationship between mating trial time and sperm availability when males mated with conspecific females (Fig. 2, linear regression: R^2=0.056, $F_{1,78}$=4.649, P=0.034). There was no significant relationship between mating trial time and sperm availability when males mated with Amazon mollies (linear regression: R^2= 0.003, $F_{1,78}$=0.202, P=0.654).

Discussion

In experiment 1, we found that male sailfin mollies had more sperm available after mating with female sailfin mollies than after mating with Amazon mollies. These results are surprising considering male sailfin mollies produce more sperm in the presence of chemical and visual stimuli of female sailfin mollies than in the presence of stimuli of Amazon mollies (Aspbury and Gabor 2004b). There are several non-mutually

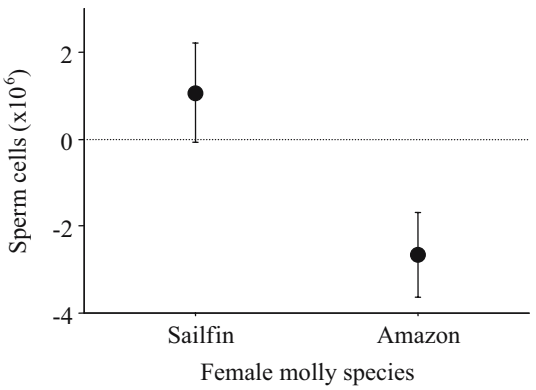

Fig. 1 Sperm availability after mating with female sailfin mollies and Amazon mollies. Male sailfin mollies, *Poecilia latipinna*, had more sperm available after mating with female sailfin mollies (mean±SE= (1.1±1.1)×10^6 cells; N=24) than after mating with Amazon mollies, *P. formosa* (−2.7±1.0)×10^6 cells; N=24). Sperm availability equals the difference between sperm extracted after mating and the day 9 measure of sperm

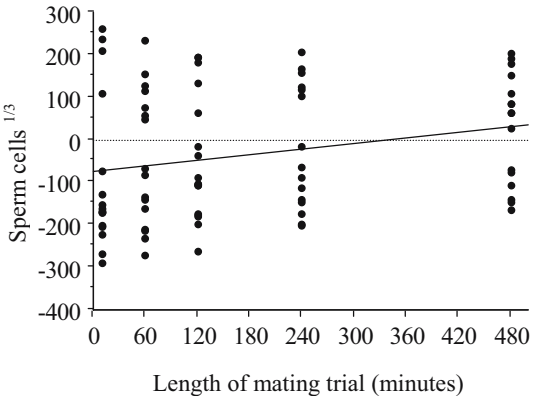

Fig. 2 Sperm availability over time in the sailfin molly, *Poecilia latipinna*. Sperm availability equals the difference between sperm extracted after mating and the day 6 amount of sperm. Males were paired with one conspecific female for 10, 60, 120, 240, or 480 min

exclusive explanations for why male sailfin mollies had more sperm available after mating with a conspecific than when mating with a heterospecific female. In this study, a positive value of sperm availability is due to our recovery of greater numbers of sperm cells from males immediately after the male has mated than the amount of sperm a male produces without physical contact with females. In experiment 1, male sailfin mollies had positive sperm availability after mating with female sailfin mollies but not after mating with Amazon mollies. One hypothesis that explains this observation is that the positive measure of sperm availability indicates that mature sperm become available (i.e., spermiation) during the mating trials due to stimulation from physical interactions with conspecific females. In poeciliid fishes, spermiation can be influenced over short time periods by environmental factors (Constantz 1989). Male sailfin mollies produce more ready sperm in the presence of female sailfin mollies than in the presence of Amazon mollies over a long period of time (7 days; Aspbury and Gabor 2004b). The results of our experiment 2 demonstrate that sperm availability did increase as trial time increased when males mated with conspecific females, but not when males mated with Amazon mollies. These results indicate that the rate of increase of sperm availability outpaced the rate of sperm expenditure only when males mated with conspecific females. Increased sperm availability during mating with conspecific females has at least two possible benefits to males: (1) males reduce physiological costs associated with spermatogenesis. Males may conserve energy by only releasing ready sperm when it is needed, rather than maintaining high levels of ready sperm at all times, and (2) males will have more sperm available and may be better able to numerically compete with other males for fertilization of eggs (Roche et al. 1968). This is particularly important for male sailfin mollies, as female sailfin mollies mate multiply and can store sperm for up to several months (Constantz 1989). In addition, males respond to sperm competition risk in this system by producing more sperm before mating, and expending more sperm during mating, when in the presence of a male competitor than when in the absence of a male competitor (Aspbury 2007).

Another explanation for the results of experiment 1 is that males were not given the opportunity to mate simultaneously with females of both species although males were in the presence of both species before mating. In this design, at the time of mating the males were essentially in a "no choice" situation. In green tree frogs, *Hyla cinerea* and *H. gratiosa*, females respond to calls of heterospecific males during no choice tests, although they prefer the call of conspecific males (Gerhardt 1974; Oldham and Gerhardt 1975). Furthermore, female sailfin mollies exhibit stronger preferences for larger males during simultaneous presentation than during sequential presentation (MacLaren and Rowland 2006). Future experiments should assess how simultaneous presentation, sequential presentation and no choice experimental designs affect sperm allocation strategies.

Another explanation for why males had higher sperm availability after mating with conspecific females than with heterospecific females in experiment 1 is that exposure to different types of females may make the mature sperm bundles more (or less) easily usable for the male and consequently strippable by the operator. We feel confident that our stripping techniques do recover most of the sperm that is available from males. Regardless, the conclusions about the adaptive value of our observations would not change even if exposure to different females makes the mature sperm bundles more available to the male. Finally, it is possible that female behavior had the potential to prevent copulation and thus reduce the amount of sperm expended by males during experiment 1. However, no obvious differences in avoidance behaviors were observed between female sailfin mollies and Amazon mollies in these experiments (DM Robinson personal observation).

In another study examining sperm transfer by male *P. mexicana* (another species that is sexually parasitized by Amazon mollies), males transferred more sperm to female conspecifics than to Amazon mollies (Schlupp and Plath 2005). The difference between our results and Schlupp and Plath (2005) may be because we measured sperm availability after mating, whereas Schlupp and Plath (2005) measured the amount of sperm recovered from the female reproductive tract after mating. In our experiments, we determined male sperm availability after mating as an estimator of sperm expenditure rather than sperm numbers recovered from mated females' reproductive tracts. Although sperm transferred to females may indicate a male's potential insemination success at a specific mating, male sperm availability after a single mating is a more accurate measure of a male's ability to inseminate females during future matings. This is especially important when the study species may encounter

many females sequentially in a given (short) period of time, as is the case for sailfin mollies. In addition, sperm loss may occur during sperm transfer, and therefore sperm recovered from females after a single mating does not necessarily reflect male sperm expenditure (e.g., Evans et al. 2003). Previous laboratory studies using poeciliids that recovered sperm from the female reproductive tract often recover a low quantity of sperm from females and/or are unable to recover sperm from many mated females (e.g., sailfin mollies: Schlupp and Plath 2005; Amazon mollies: Schlupp and Plath 2005; eastern mosquitofish, *Gambusia holbrooki*: Evans et al. 2003).

Our results illustrate the potential problems associated with measuring male sperm expenditure. We suggest that the choice of response variables may be very important when testing hypotheses regarding male sperm allocation. If a researcher is interested in questions related to a female's fitness from mating with particular males (e.g., Pilastro et al. 2002), then the use of sperm recovered from a female's reproductive tract is likely the best variable to measure, assuming that the recovery of sperm cells from a female is repeatable. The same argument holds true if the researcher is interested in the potential reproductive success of a male at a given mating event. Alternatively, we suggest that if the focus of the study is to examine a male's potential future reproductive success, then measures of sperm recovered from a female's reproductive tract are not as appropriate as measures of sperm availability after a male is mated. Our results demonstrate that a male's sperm availability can be affected at a very small temporal scale by social stimuli. By examining sperm availability after mating, we have shown that measuring effects of heterospecific mating on male fitness may be much more complex than previously thought.

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