

Male mating behavior and ejaculate expenditure under sperm competition risk in the eastern mosquitofish

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Theory predicts that males should tailor the size of their ejaculates according to temporal changes in the risk of sperm competition. Specifically, males are predicted to allocate more sperm to each mating event with increasing risk (i.e., the probability that the sperm from two males will compete for fertilization). We tested this hypothesis by using the eastern mosquitofish, a freshwater species of fish exhibiting a coercive mating system and internal fertilization. We manipulated the perception of sperm competition risk by adjusting the sex ratio under which males were maintained over 8 days. Males were housed either with three females and one male (simulating high sperm competition risk) or with four females (low risk). After the treatment, we presented each test male individually to an unfamiliar male-deprived female for 30 minutes and observed his mating behavior. We then artificially stripped the test males of sperm and recovered the ejaculates from the females. Our results revealed that males in the high-risk group performed higher levels of mating activity and sperm expenditure (i.e., used up more of their sperm reserves) than did low-risk males. A control experiment, in which test males were treated but did not participate in the mating trials, revealed no significant difference in the number of sperm stripped from high- and low-risk males, indicating that sperm production was not affected by the treatment. We did not detect a difference in the number of sperm retrieved from females among the groups, raising the possibility that some sperm are lost during mating activity, either through ejaculation with incomplete or interrupted penetration, or via female ejection. *Key words:* coercive matings, guppy, Poeciliidae, sperm competition theory, sperm store. [*Behav Ecol* 14:268–273 (2003)]

Sexual selection will favor any adaptation in males that increases their success in sperm competition, defined as the contest between the sperm from two or more males for the fertilization of a set of eggs (Parker, 1970). When sperm compete on a numerical basis, the most evident adaptation to sperm competition is the production and subsequent ejaculation of numerous sperm (Parker, 1982). However, ejaculates can be energetically costly to produce (Dewsbury, 1982; Nakatsuru and Kramer, 1982; Olsson et al., 1997; Shapiro et al., 1994), and males are expected to strategically allocate sperm in response to varying levels of sperm competition (Parker, 1990a). Specifically, theory predicts that ejaculate expenditure should depend on the number of males competing for fertilization (Parker et al., 1996, 1997). When there is low probability of competition between a maximum of two ejaculates, male gametic expenditure is predicted to increase with sperm competition risk (Parker et al., 1997). Support for this prediction comes from comparative studies spanning several taxonomic groups (Gage, 1994; Harcourt et al., 1981; Hosken, 1997; Møller, 1988, 1991) and from within-species studies, including insects (Cook and Wedell, 1996; Gage, 1991; Gage and Barnard, 1996; Simmons and Kvarnemo, 1997; Simmons et al., 1993) and recently birds (Hunter et al., 2000; Nicholls et al., 2001).

In fishes, in which the majority of sperm competition studies have focused on externally fertilizing group-spawning species (for review, see Petersen and Warner, 1998), interspecific patterns of male gametic investment have been shown to co-vary with sperm competition intensity in accordance with theoretical predictions (Stockley et al.,

1997). Studies that have focused on within-species patterns of sperm production have tended to examine groups in which males exhibit alternative mating tactics (Gage et al., 1995; Gross, 1982; Petersen, 1990; Pilastro and Bisazza, 1999; Robertson and Warner, 1978; Warner and Robertson, 1978) rather than on those in which individual males face varying levels of sperm competition among a series of spawns (Fuller, 1998; Petersen and Warner, 1998). Furthermore, no studies have examined the ejaculation strategies of species exhibiting internal fertilization, even though they are ideally suited for such studies. Unlike in external fertilizers, in which the assessment of sperm competition risk can be problematic owing to the difficulty in measuring the number of males actually participating in a spawning event, it is relatively easy to both manipulate and assess the level of sperm competition in internal fertilizers (see Petersen and Warner, 1998).

Previous studies on sperm competition in internally fertilizing fish have focused almost exclusively on one family, the Poeciliidae (see Constantz, 1984; Evans and Magurran, 2001; Hildemann and Wagner, 1954; Pilastro and Bisazza, 1999; Zane et al., 1999). Poeciliids are a group of live-bearing fish, native to the New World but introduced elsewhere as mosquito control agents and ornamental fish. Among these, the eastern mosquitofish *Gambusia holbrooki* was introduced into Italian fresh and brackish waters at the beginning of last century (Dulzetto, 1928). As in other poeciliids, fertilization in eastern mosquitofish is internal, and during copulation, males transfer sperm in bundles (spermatozeugmata) by inserting their modified anal fin, the gonopodium, into the female's genital pore (Constantz, 1989). Shortly after insemination, the sperm bundles break apart in the gonoduct, and spermatozoa not immediately used for fertilization can be stored for several months (Dulzetto, 1928). Females are ovoviviparous, retaining yolked embryos without nourishing them (Constantz, 1989). Unlike some poeciliids, in which individual males display alternative mating tactics (for review,

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see Meffe and Snelson, 1989), male eastern mosquitofish appear to exhibit no courtship and achieve inseminations through gonopodial thrusts (Bisazza, 1993; McPeck, 1992). High levels of multiple paternity within the broods of the eastern mosquitofish (Zane et al., 1999), coupled with the females' ability to store sperm (Constantz, 1984, 1989), suggests that sperm competition is intense in the species.

In the present study, we investigate the effect of varying sperm competition risk on the mating behavior and sperm expenditure of male *G. holbrooki*. As Parker (1998) notes, engaging in repeated copulations toward individual females is a means by which males can adjust sperm allocation in response to changes in the risk of sperm competition. This strategy is likely to be especially important when males are limited in their ability to tailor the size of individual ejaculates during a single copulation (see Birkhead and Møller, 1992). Such a constraint is likely to apply to male *G. holbrooki*, in which gonopodial thrusts, each lasting a fraction of a second, are performed repeatedly towards individual females. Consequently, there is likely to be minimal scope for adjusting ejaculate size during individual gonopodial thrusts in this species. Accordingly, we anticipated a general increase in the frequency of coercive mating attempts by males, resulting in increased sperm allocation and higher levels of sperm expenditure under elevated sperm competition risk. Incidences in which spermatozeugmata are released into the water during male mating attempts have been observed in mosquitofish (Evans JP, Pierotti M, Pilastro A, unpublished observations). We therefore considered as a measure of sperm expenditure (allocation) both the number of sperm inseminated and the sperm reserves left after the experiment.

METHODS

The study population and its maintenance

Eastern mosquitofish were collected early in the breeding season (which lasts from May until September) from Valle Avertio, a system of brackish water ponds and ditches in the Venetian lagoon basin (northern Italy). Early in the season, the sex ratio of natural populations is typically biased toward females owing to differential male–female survival over winter (Zulian et al., 1995). During the course of the breeding season, sex ratios become increasingly male-biased (Zulian et al., 1995). The fish were returned to the laboratory and maintained in mixed-sex groups in several stock aquaria (150 l, provided with natural gravel and an air filter) until they were used for the experimental trials. These aquaria were partially divided by green comblike plastic barriers simulating plants. We also maintained separate tanks containing male-deprived females for the mating trials ($n = 120$ females isolated from males for 45 days). Depriving females of males for this period ensured that any sperm extracted from their gonoducts after the mating trials were inseminated by the test male and not by previous mating partners (Pilastro et al., 1997). Water temperature was maintained at $25^\circ \pm 2^\circ\text{C}$ and fish were fed to satiation each morning with commercially prepared flake food (Sera GVG-mix).

Sperm competition risk treatment

Sexually mature males, recognizable by their fully developed intromittent organ (the gonopodium), were chosen randomly from several stock tanks and placed for 8 days in 90-l treatment tanks containing either (1) four females (stimulus fish to simulate low-sperm competition risk) or (2) three females and a male (simulating high risk). The two sex ratios fall within the range naturally encountered by males in the

wild, and are known to change over similar time scales to the one chosen for our study (Zulian et al., 1995). Following the 8-day treatment period, focal males were moved into the experimental tanks for the mating trials (see Mating Trials below), and the stimulus fish were returned to stock aquaria and replaced by others for the next trials. All fish (focal and stimulus) were assigned randomly to each experimental group, and tanks were randomized with respect to treatment. Treatment tanks contained natural gravel, air filters, and green plastic comblike barriers designed to create a heterogeneous environment for the fish. Sample sizes for the experiment were $n = 31$ (low risk) and $n = 25$ (high risk).

Mating trials

Focal males were individually placed into one of four experimental tanks ($35 \times 45 \times 40$ cm, filled to a depth of 30 cm) and left to acclimatize overnight. Attached to each of these tanks was a second smaller tank ($20 \times 15 \times 15$ cm) in which we placed stimulus fish designed to maintain each focal male's perception of sperm competition risk during the acclimation period. In each experimental tank containing a "high-risk" male, the small tank contained three females and a male (unfamiliar to the test male). Likewise, in the low-risk tanks, we placed four females in the small enclosure. We ensured that the test males had visual but not olfactory access to the stimulus fish. Treatments were randomized among the four experimental tanks to avoid possible bias.

On the following morning, the small tanks containing the stimulus fish were carefully removed from the experimental aquarium. We then placed a male-deprived female (who was consequently naïve of the experimental treatment) into the tank with the male and began our behavioral observations as soon as the male performed his first mating attempt. These were characterized by the male approaching the female with his gonopodium swung forward (more than 90° angle) and his snout drawing level or beyond the tip of the female's caudal fin (Pilastro et al., 1997). We also recorded the number of genital contacts between males and females, which were highly conspicuous owing to the female's "jolting" response when the male's gonopodium made physical contact with her genital region. Mating trials lasted 30 min (from the first mating attempt), and to estimate male mating behavior, we considered the number of mating attempts and genital contacts performed during the initial 20 min of each trial. All behavioral observations were performed blind of experimental group (i.e., the observer was not aware of the test male's treatment status), and each trial was video-recorded to aid our subsequent analysis of male behavior. Mating trials took place between 0900 and 1400 h.

Control experiment

The time required for full sperm replenishment has not been investigated in eastern mosquitofish, but observations in guppies (*Poecilia reticulata*) suggest that males can restore their sperm reserves in 1 day (Kuckuck and Greven, 1997). Nonetheless, in a separate experiment, we controlled for the possibility that test males in the 2 experimental groups entered the mating trials with different-sized sperm reserves, for example, as a result of differential sperm production or mating activity among the two treatment groups. We performed a series of identical trials to those described above in which control males were treated for 8 days (high-risk, $n = 14$; low-risk, $n = 13$) but did not participate in the mating trials. Instead, these males were allowed to rest overnight (as in the experimental groups) and were then artificially stripped of sperm (see below). Sperm counts were then

compared among males from both groups to control for the potentially confounding effect that males entered the mating trials with different sized sperm reserves.

Sperm counts

Within 10 min of the behavioral trials, test males and females were isolated, anaesthetized with MS222, and placed on a Petri dish under a dissection microscope. For each male, standard length (SL) was measured to the nearest 1 mm. To collect sperm from males, the gonopodium was swung forward to induce the formation of a temporary groove through which sperm bundles could pass; gentle pressure applied to the side of the abdomen, at the base of the gonopodium, induced sperm release (Constantz, 1984; Peden, 1972). After repeating this action to remove all sperm bundles, the stripped ejaculate was recovered under low magnification by using a glass micropipette and diluted in 100 μ l of physiological solution (0.9% NaCl).

To collect ejaculates from the females, approximately 10 μ l of physiological solution was taken from a microtube containing 50 μ l of the solution and injected into the female's gonoduct by using a glass micropipette (Pilastro et al., 1997). Samples were retrieved from the female and returned to the microtube. We repeated this process three times to ensure that we collected all recoverable sperm from the females. All samples (male and female) were subsequently drawn up and expelled from the pipette to aid the breakdown of bundles and to produce an even dispersal of sperm cells. The number of sperm per stripped and natural ejaculate was estimated by using a Bürker counting-chamber under $\times 400$ magnification. After checking that sperm cells were evenly distributed across the chamber, sperm number was determined by multiplying the mean from five sperm counts by the sample's dilution factor and initial volume (see Matthews et al., 1997). As in the mating trials, sperm counts were performed blind of experimental group in order to avoid possible observer bias. We confirmed that the SL of males, females, and control males did not significantly differ between the treatment groups (test males: $t_{53} = 0.22$, $p = .83$; test females: $t_{53} = .41$, $p = .69$; male SL/female SL: $t_{53} = 0.21$, $p = .84$; control males: $t_{53} = 1.13$, $p = .27$). After the extraction of sperm, males and females were revived in conditioned fresh water (Stress Coat, Aquarium Pharmaceuticals Inc., Chalfont, Pennsylvania, USA) and returned to stock aquaria, where they played no further part in the experiment.

Analysis

Statistical analyses were performed by using SPSS 10.1 (Norusis, 1993). When data were not normally distributed, we used either log transformation or nonparametric tests. In the latter case, exact probability is given. Because male body size is positively correlated with the number of sperm stripped and negatively correlated with the number of male mating attempts (Bisazza and Marin, 1995; Pilastro et al., 1997), we controlled for male size by using an ANCOVA in which treatment was the factor; male SL, the covariate; and male mating attempts or sperm stripped, the dependent variables. In one case (sperm stripped), the variances in the two groups were not homogeneous, even after log transformation. We therefore compared the residuals of the regression of sperm stripped on male length by using a Student's t test for non-homogeneous variances, and reduced the degrees of freedom by one to account for having used residuals (Norusis, 1993). If not otherwise stated, mean \pm SE is given. All probabilities are two-tailed. We failed to measure SL of one male and one female and to strip sperm from one male from

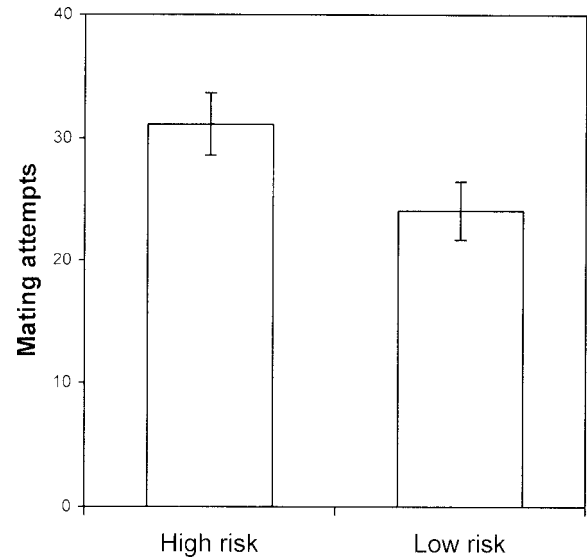


Figure 1
Number (mean \pm SE) of mating attempts performed by male *G. holbrooki* during 20-min behavioral trials after the 8-day treatment period.

the low-risk group. Sample sizes can therefore differ between analyses.

RESULTS

Males assigned to the high-risk group performed significantly more mating attempts than those in the low-risk treatment ($t_{54} = 2.15$, $p = .036$; Figure 1). To remove the effect of male body size, which was negatively correlated with mating frequency ($r = -.28$, $n = 55$, $p = .04$), we performed an ANCOVA with male SL as the covariate. This analysis confirmed that high-risk males performed more mating attempts than did their low-risk counterparts during the 20-min observation period (model: $F_{2,52} = 4.64$, $p = .014$; male SL: $F = 4.98$, $p = .03$; treatment: $F = 4.58$, $p = .037$). The mean number of genital contacts was slightly higher in the high-risk group, but the difference was not significant (high-risk males: mean = 3.56 ± 0.42 , $n = 25$; low-risk males: mean = 3.29 ± 0.36 , $n = 31$; $t_{54} = .49$, $p = .63$).

Our analysis revealed that the number of mating attempts was positively correlated with the number of sperm inseminated among males from both treatments (Spearman rank correlation: $r_s = .40$, $n = 56$, $p = .002$). However, we found no significant difference in the number of sperm retrieved from females among the two treatment groups (high-risk treatment: mean = $0.03 \times 10^6 \pm 0.12$, $n = 25$; low-risk treatment: mean = $0.03 \times 10^6 \pm 0.19$, $n = 31$; $U = 364$, $p = .61$, Mann-Whitney U test). In most cases, we did not retrieve sperm from the female (high-risk group = 18/25; low-risk = 24/31); the frequency of females without sperm did not differ between groups (Fisher's exact test: $p = .76$). When we further categorized males within each treatment group according to whether they successfully inseminated the female (i.e., based on whether sperm were retrieved from the female), we found that successful males ($n = 14$) performed significantly more mating attempts than did those who failed to inseminate the female ($n = 42$, $t_{54} = 3.03$, $p = .004$; Figure 2). In addition, successful males obtained more genital contacts than did unsuccessful ones ($n_1 = 14$, $n_2 = 42$, $U = 172.5$, $p = .016$).

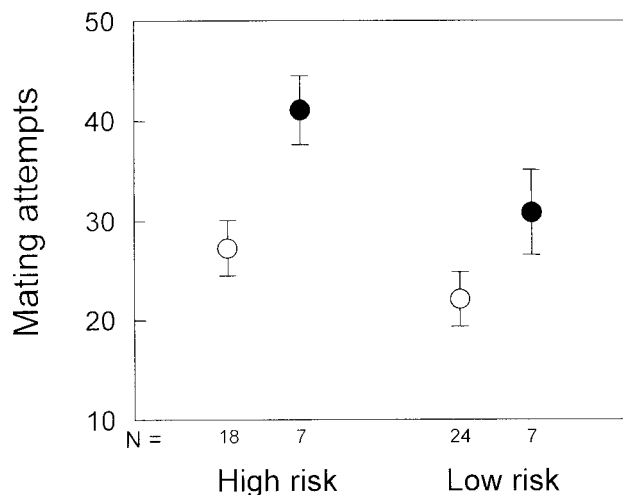


Figure 2
Number (mean \pm SE) of mating attempts performed by male *G. holbrooki* according to treatment (high or low sperm-competition risk) and insemination status of the female. Within each treatment, males were classified according to whether they successfully inseminated (filled circles) or failed to inseminate the female (open circles) during the 20-min mating trials.

Our analysis revealed that high-risk males stripped fewer sperm after the mating trials than their low-risk counterparts (analysis by using residuals of the regression between body size and the number of stripped sperm: $t_{51} = 2.26$, $p = .035$; Figure 3). This finding was supported by the results from the control experiment, which confirmed that high- and low-risk males did not enter the mating trials with different-sized sperm reserves (mean sperm counts: high-risk males = $1.07 \times 10^6 \pm 0.36$, $n = 14$; low-risk males = $1.07 \times 10^6 \pm 0.87$, $n = 13$; $t_{25} = 0.91$, $p = .37$, after log transformation). There was no significant difference in the number of sperm stripped between the two groups in the control experiment even after controlling for male size (ANCOVA, model: $F_{2,26} = 4.10$, $p = .029$; male SL: $F = 7.17$, $p = .013$; treatment: $F = 0.014$, $p = .91$). Some males did not strip any sperm in the control experiment (Fisher's exact test: high-risk = 5/14; low-risk = 3/13; $p = .68$), and when these males were removed from the analysis, high-risk males stripped significantly more sperm than did low-risk ones ($t_{18} = 3.17$, $p = .005$). This further supports our conclusion that in the experimental treatments, sperm expenditure during the mating trials by high-risk males was greater than by the low-risk group.

DISCUSSION

Our results indicate that male eastern mosquitofish strategically allocate both mating effort and sperm output in response to varying sperm competition risk (as simulated by variation in the sex ratio). Males in the high-risk group performed significantly more mating attempts than did those assigned to the competition-free group. Furthermore, our finding that the number of sperm per stripped ejaculate was significantly lower in high-risk males after the mating trials suggests that they respond to the elevated risk of sperm competition by increasing their expenditure on sperm. This conclusion was supported by the results from the control experiment, which confirmed that sperm production was not (significantly) affected by the treatment. A recent study has confirmed that sperm production is unaffected by sperm competition risk even when the treatment period is extended

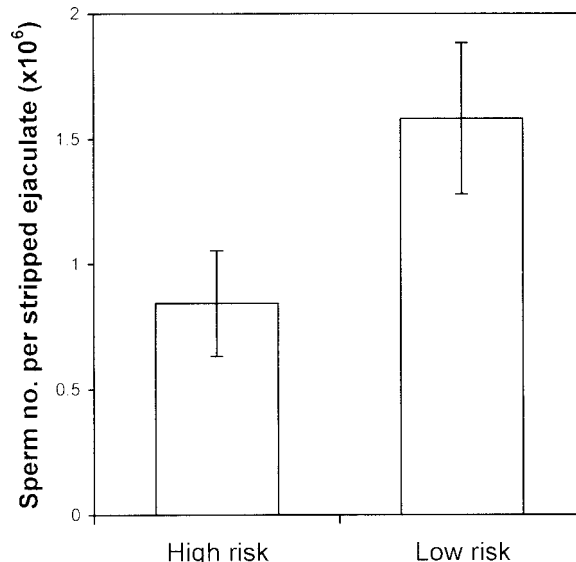


Figure 3
The number (mean \pm SE) of sperm stripped from males at the end of the mating trials according to treatment status.

to 16 days (Evans JP, Pierotti M, Pilastro A, unpublished observations).

To our knowledge, this is the first evidence that individual males vary their sperm expenditure according to sperm competition risk in an internally fertilizing species of fish. In other studies, empirical support for strategic ejaculation comes mainly from insects (see Simmons and Siva-Jothy, 1998, and references therein). For example, in the Indian meal moth (*Plodia interpunctella*), males strategically allocate sperm in response to the presence and size of rival sperm bundles (spermatophores) in the female reproductive tract (Cook and Gage, 1995). Two recent studies confirm that male birds also tailor the size of their ejaculates in response to their perception of sperm competition risk (Hunter et al., 2000; Nicholls et al., 2001).

Importantly, our analysis revealed that higher mating effort by males increases the likelihood of successful insemination. When we categorized females according to whether sperm were retrieved from their gonads, we found that in "successful" trials, males performed more mating attempts than did unsuccessful ones, irrespective of treatment group, which also affected mating effort (see Figure 2). This strongly suggests that male mating success (in terms of sperm transfer) is influenced by the number of attempts made, rather than on the number of sperm allocated per mating attempt (see discussion by Parker, 1998). However, despite the differences in mating effort among the groups (and its subsequent advantage in terms of insemination success), our results indicated that few males succeeded in inseminating the female. This suggests that males waste part of their sperm output during gonopodial thrusts. Indeed, in the mating trials, and during our subsequent observations of video footage taken during the trials, we noted several incidences in which spermatozeugmata were released into the water. Unfortunately, it was not possible to determine whether these sperm bundles were ejected by females or were instead the result of ejaculations with incomplete or interrupted penetration by males. It would be interesting to determine the frequency of contacts that result in sperm loss because it may represent a substantial energetic cost for the male. An intriguing possibility is that sperm transfer is partially under

female control, for instance, via the selective rejection (Eberhard and Cordero, 1995; Pizzari and Birkhead, 2000) or replacement (see Constantz, 1984) of spermatozoa after forced copulations. This possibility clearly warrants further investigation.

Other plausible explanations to account for low insemination success uncovered by our study include the possibility that our extraction method was unreliable. However, previous work has confirmed that it is possible to extract sperm from recently mated female mosquitofish by using identical techniques to the ones used here (Giacomello, 1995; Pilastro et al., 1997). In addition, it is possible that the 30-min mating trials provided insufficient time for all males to achieve successful insemination. Although extending the duration of the mating trials may have resulted in a higher proportion of females being inseminated (Giacomello, 1995), the effect of the treatment may have been weakened or diminished if we had chosen considerably longer trial periods. Moreover, the number of recoverable sperm from the gonoduct of female poeciliids is known to decrease over time (Constantz, 1984), although the precise rate of decline is presently unknown.

According to our results, male eastern mosquitofish tune their mating behavior in response to the sex ratio they have experienced previously rather than by instantaneously assessing the number of competitors at the time of mating. In our experiment, each experimental male was paired with a single female during the mating trials and therefore would have perceived low sperm-competition risk during the mating trials. We chose this design both because of its biological relevance (see below), and because it avoided the confounding influence of differential sex ratio among the experimental treatments at the time of mating. We therefore avoided the possibility that male behavior and ejaculate expenditure was partly influenced by interactions between the female with whom he had mated (who in our study was naïve of the experimental treatment) and a stimulus male (in the case of the high-risk treatment). In addition, our experimental design was biologically realistic because male eastern mosquitofish do not participate in stable schools but instead move from one group of females (usually two to five females) to another (Zulian et al., 1995). This makes it impossible for them to instantaneously gauge a given female's past and future mating opportunities (see Parker, 1998, p. 21 for discussion), and means that a male's best strategy is to assess the average level of sperm competition in the population and adjust his mating effort accordingly. Such a strategy is likely to be important in *G. holbrooki*, in which sex ratios in wild populations are known to vary predictably over the course of the breeding season in northern Italy. Typically, at the start of the season, sex ratios are biased toward females because of differential over-winter mortality among the sexes (Zulian et al., 1995; for discussion, see Snelson, 1989). However, population sex ratios in mosquitofish become increasingly male biased as the breeding season progresses, presumably because of differential growth rates and predation pressure among the sexes (Snelson, 1989; Zulian et al., 1995).

Although we interpret our results here in the context of sperm competition theory, qualitatively similar results would be expected if males place higher reproductive value to each mating attempt when females are relatively scarce in the population (i.e., similar to our high-risk treatment). In this context, males may also allocate greater mating effort when encountering females. Further work, in which males are exposed to varying numbers of females (but in the absence of a competing male), may help to distinguish between these two alternative hypotheses, although density effects on subsequent male mating behavior may confound the interpretation of the results.

In conclusion, we provide empirical support for Parker et al.'s (1997) risk model of sperm competition by showing that male eastern mosquitofish tailor their mating behavior and ejaculate expenditure according to variation in the sex ratio. Further investigations are warranted in order to determine whether males also adjust their behavior and ejaculate expenditure in response to variation in the intensity of sperm competition (i.e., when more than two ejaculates compete) in accordance with theoretical predictions (Parker et al., 1996).

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