

Sexual selection for small size in male mosquitofish (*Gambusia holbrooki*)

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SUMMARY

In many poeciliid fishes, large males which court females coexist with small males which sneak-copulate. It is unclear whether these two tactics represent two evolutionarily stable strategies or if sneaking is a conditional strategy adopted by small, unattractive males. We studied the success of sneaky copulation by looking for sperm in the gonoduct of females after they were kept for 48 hours with a male. A logistic regression analysis showed that the probability of a female being inseminated increased with female length and decreased with male length. The length of the males relative to that of the females was the best predictor of success. This result was confirmed using virgin females, thereby excluding any possible confounding effect due to the release of sperm from previous copulations. Sperm counts suggested that large males do not compensate for their reduced copulatory success by releasing larger sperm numbers. Behavioural data indicate that the advantages to small males are twofold: they have a greater chance to approach females from behind without being detected, and manoeuvre better when inserting the gonopodium into the female's gonoduct. The selective advantage of small size might explain male dwarfism in poeciliids. Our results also suggest that small males adopting the sneaky tactic may be as successful as large males adopting courtship, and that alternative mating strategies may be maintained by negative density-dependent selection.

1. INTRODUCTION

Females are usually very selective in their mate choice, and reluctant to copulate with most males encountered, since only one or few matings are enough to fertilize all their gametes. Not surprisingly, sexual coercion has evolved in many animal species as an alternative to attracting females by courtship. In many instances, males make use of their greater physical strength to force copulations, to sequester females or to harass them until they obtain a copulation (Clutton-Brock & Parker 1995). Force is not always necessary to bypass the female's consent: in species with external fertilization, males can approach a mating pair and release their sperm at the time of spawning. Sneaking behaviour is usually favoured by inconspicuousness, i.e. a small body size can be advantageous for the male (Gross 1985).

Another way to sneak copulations is observed in poeciliids and in a few other fishes with internal fertilization (Kulkarni 1940; Farr 1989). The male approaches a female, usually from behind, and tries to thrust his gonopodium into the female's gonoduct or to attach sperm near her genital pore. In the last two decades, poeciliids have become a preferred subject in studies on sexual

selection, in particular concerning female choice and the evolution of sexual secondary characters (e.g. Ryan & Wagner 1987; Houde & Endler 1990). Conversely, sneaky copulation has received much less attention, despite the fact that it is the most common mating tactic within poeciliids (Farr 1989).

A second poorly known aspect of the biology of poeciliids is the nature of the selective forces acting on body size in the two sexes. It is unclear how polymorphism in male size is maintained in natural populations and what causes the extreme smallness of males in many species. In the sailfin molly, *Poecilia latipinna*, the swordtail, *Xiphophorus nigrensis*, and in several other poeciliids, males show considerable variation in body size, associated with differences in mating behaviour. Typically, large males exhibit bright coloration and court females, whereas small males are inconspicuous and attempt to sneak-copulate. In these species, sneaky copulation has been commonly regarded as 'the best of a bad job' tactic employed by smaller, unattractive males (Clark *et al.* 1954; Ryan & Wagner 1987; Zimmerer & Kallman 1989). However, evidence from many poeciliids now indicates that male body size, as well as the mating tactic exhibited, is genetically controlled (Zimmerer & Kallman 1989; Erbelding *et al.* 1994; Travis *et al.* 1997). This raises the question of how alleles for small body size are maintained despite the advantage of large males in both male-male competition and female choice (Farr *et*

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al. 1986; Zimmerer & Kallman 1989; Erbelding *et al.* 1994).

In most poeciliids, males are usually much smaller than females, some species being among the most extreme examples of reversed size dimorphism in vertebrates. Many explanations have been proposed to explain such pronounced size dimorphism (see Bisazza (1993) for a review), but none has yet been tested. In all species studied so far, the females actively tries to avoid sneaky copulation by escaping, by adopting positions preventing copulation or, more rarely, by directly attacking males. Therefore, the male approaches a female from behind and tries to remain within the blind portion of her visual field. Some authors have suggested that small males, being relatively inconspicuous and more agile, may be more successful than larger males by using this tactic (Hughes 1985). If so, in polymorphic species, alleles for small body size may be maintained because the mating success of the two genotypes is frequency dependent (Constantz 1975; Bisazza 1993). A small male advantage in sneak-copulating might explain why males are so small, compared to females, in those species where males adopt only or mainly this tactic.

In the eastern mosquitofish, *Gambusia holbrooki*, males rely mostly on sneaking to copulate. A previous ethological study (Bisazza & Marin 1995) suggested that small males are more likely than large males to achieve a contact between genitalia during a sneaky mating attempt. The success of attempts increased with the length of the female and decreased with the length of the male. However, since gonopodial thrusting lasts less than one second, it is impossible to determine from purely behavioural observations whether sperm transfer actually occurs.

Here, we studied pairs of mosquitofish and measured the frequency of females inseminated in relation to the body size of the fish. As the small male advantage in sneaking copulations could be associated with either a reduced conspicuousness or to a greater manoeuvrability, we carried out behavioural observations on male sneaky copulatory attempts to assess the relative importance of these components in determining the success of small males.

2. MATERIALS AND METHODS

(a) *Experiment animals and insemination assay*

Fish used in the experiments derived from a feral population near Padua (Italy). They were stocked in large aquaria kept at a constant temperature ($26 \pm 1^\circ\text{C}$) and photoperiod (06.00 to 20.00 hours). Fish were fed *ad libitum* twice a day with dry food. At the end of each trial, males and females were anaesthetized with MS222 and their standard length (SL) was measured to the nearest 0.5 mm. The female was then checked for the presence of sperm, according to Clark *et al.* (1954): using a glass micropipette, a small volume of physiological solution (NaCl 0.9%) was injected into the female's gonoduct. The solution was drained and the number of sperm was determined with a Bürker haemocytometer, using a microscope ($\times 250$ magnification). The total number of sperm was counted up to 20 sperm per square of the haec-

cytometer grid. With higher densities, their number was estimated from the number of sperm in a minimum of ten squares. After being tested, fish were allowed to recover for some days and then released back into the wild.

After a copulation, sperm are found in the oviduct for up to one week, and after two weeks none was ever found (Clark *et al.* 1954; Liley 1966; Giacomello 1995). However, like most poeciliids, female mosquitofish store sperm for several months in folds lining the ovary (Constantz 1989) and some sperm could pass in the oviduct. In order to test for that, we carried out the following tests. Twenty-one females, previously kept with males, were deprived of males for at least 15 days, and then checked for the presence of sperm (control 1a). A second group of 21 females were kept with males for the same period and then checked for the presence of sperm (control 1b).

(b) *Insemination success in relation to male and female body size*

One male was placed in a 165 l aquarium, containing plants and rocks, and allowed to settle overnight. One female, previously deprived of males for at least 15 days, was introduced into the aquarium and allowed to stay for 48 h. Eighty-five replicates were run. Pairs were formed such as to cover the whole range of variation of male/female relative size (Bisazza & Marin 1995) (table 1). Female deprivation time ranged between 15 and 43 days (mean = 28.8 days, s.d. = 7.2). Only females that did not deliver young in the seven days before the experiment were used. To control for possible differences in the rate of mating attempts among males, in a subset of these male–female pairs (68 replicates), the number of attempts was recorded during four 15-min periods, soon after the female was introduced into the aquarium, about 1 h later, 1 h before, and immediately before the end of the trial, respectively. Fifty more replicates were done using virgin females. Virgin females were obtained by raising laboratory-born females to maturity in large aquaria where they were separated from males. These replicates lasted 24 h.

We used a logistic regression to determine whether male insemination success was affected by male and female length. In SPSS, a stepwise procedure of logistic regression was used. Significance of the coefficients was determined using the Wald statistic, which has a χ^2 distribution (Norušis 1993).

(c) *Behavioural analysis of factors determining mating success of small males*

Sneaky attempts consist of a rather stereotyped behavioural sequence. It begins with a male orienting himself behind the female and approaching her. The male then swims underneath

Table 1. *Standard lengths of males (SLM) and females (SLF) used in the experiments: means \pm s.d. (range) are given*

experiment	<i>n</i>	SLM (mm)	SLF (mm)	SLM/SLF
gravid	85	24.8 \pm 3.6 (18.5–31.0)	32.7 \pm 5.2 (23.0–42.5)	0.78 \pm 0.17 (0.47–1.24)
virgin	50	24.8 \pm 2.7 (19.5–30.0)	28.2 \pm 3.0 (22.0–35.0)	0.90 \pm 0.18 (0.62–1.27)

the female, and after reaching her genital pore, swings the gonopodium *ca.* 180° forward, orients himself upwards, slides by the female's side, and tries to insert it into her genital pore. Small male advantage in sneaky mating attempts could be due either to a reduced conspicuousness, which is helpful in the first part of the mating attempt, or to a greater manoeuvrability, which is helpful in the second part. To study that, we analysed video recordings of sexual activity of 46 males (a total of 3196 mating attempts) kept in conditions similar to that of a previous experiment (see Bisazza & Marin (1995) for details). For each male we calculated the proportion of mating attempts in which the male's snout reached the female's tail as a measure of his ability to approach the female undetected. The ability to manoeuvre was estimated for each male as the proportion of successful mating attempts (male touching the female gonopore with his gonopodium) over the number of mating attempts in which the male had reached with his snout the female's genital pore. Statistical analyses were performed using an SPSS 6.0 for Windows package.

3. RESULTS

(a) Insemination assay

All females kept with males for at least 20 days (control 1a) contained sperm in their gonoducts (mean number of sperm = 989.5, s.d. = 2161.4; $n = 21$). Four out of 21 male-deprived females (control 1b) had traces of sperm (mean = 5.8, s.d. = 6.9, $n = 4$) in their gonoduct, whereas the others did not. The difference in deprivation time between females with sperm and females without sperm, although non-significant, was opposite to that predicted if the sperm came from recent copulations (50.3 days, s.d. = 5.9, $n = 4$ and 39.9 days, s.d. = 12.6, $n = 17$, respectively; Mann-Whitney U -test, $z = 1.33$, n.s.). The average sperm number found in the females with sperm in the control 1b group was nearly 200 times smaller than that found in females that had recently mated with males (Mann-Whitney U -test, $z = 3.11$, $p = 0.002$), and the two distributions did not overlap. The maximum sperm number (16) found in deprived females was therefore used as a threshold value of sperm number for discriminating between females inseminated during the experiments and females that may have released sperm stored from previous copulations. The results of the following statistical analyses did not change substantially when this correction was not used.

(b) Insemination success in relation to male and female body length

Smear analysis revealed that 50 out of 85 test females contained sperm in their gonoducts. Among these females, nine had less sperm than the aforementioned threshold value, and therefore were classified as non-inseminated. After correction, the estimated insemination frequency was 48.2%. The effect of female deprivation time, and male and female body size on insemination probability was studied using a multiple logistic regression, with presence/absence of sperm in the female gonoduct as the dependent variable. The probability that females were being inseminated increased with the time of separation from males prior to the experiment (Wald = 7.89,

$r = 0.224$, $p = 0.005$); insemination probability also increased with female length (Wald = 6.78, $r = 0.202$, $p = 0.009$) and decreased with male length (Wald = 4.72, $r = -0.152$, $p = 0.03$, $n = 85$). Along with deprivation time (Wald = 7.42, $r = 0.215$, $p = 0.006$), relative male length (male SL/female SL) was the best predictor of the insemination probability (Wald = 10.31, $r = -0.266$, $p = 0.001$, $n = 85$; figure 1a). These two variables allowed correct prediction of about 70% of the insemination events. The number of copulatory attempts was negatively correlated with the male's relative length in the first observation period ($r = -0.42$, $p < 0.001$, $n = 68$), but not in the following three periods ($r = -0.19$, n.s.; $r = -0.19$, n.s., and $r = -0.04$, n.s., respectively). Males were never observed courting females, and females were never observed obviously cooperating with them.

Twenty-six out of 50 (52%) virgin females contained sperm in their gonoducts after 24 h with the male. The probability of virgin females being inseminated decreased with male relative size (Wald = 6.26, $r = -0.248$, $p < 0.01$, $n = 50$, figure 1b).

Male size was not correlated with the number of sperm found either among inseminated gravid ($r = 0.13$, n.s., $n = 41$) and virgin females ($r = 0.01$, n.s., $n = 26$), or in the two groups pooled ($r = 0.05$, n.s., $n = 67$).

(c) Behavioural analysis of factors determining mating success of small males

Males were able to reach the tail of the female in 46% of the observed mating attempts. The proportion

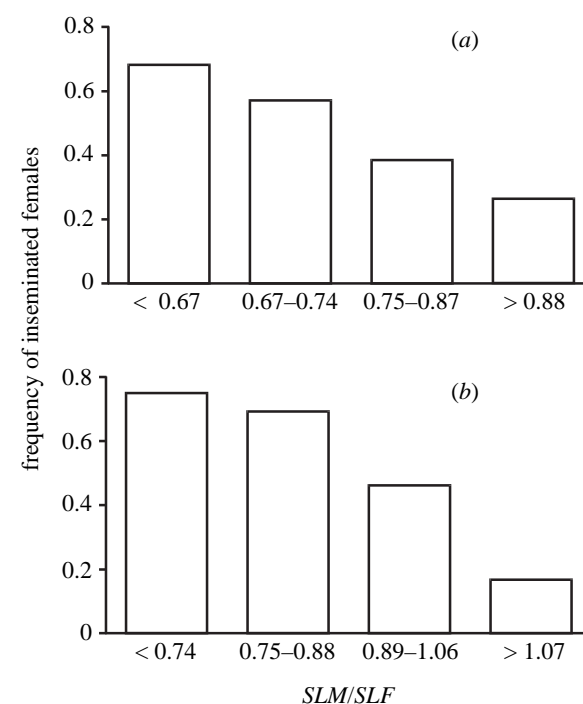


Figure 1. Frequency of female mosquitofish that were inseminated in relation to the males' relative length (male standard length/female standard length). (a) gravid females ($n = 85$); (b) virgin females ($n = 50$). Data were grouped according to quartiles.

of successful approaches achieved by each male was negatively correlated with his size and positively correlated with female size (multiple regression, female SLF, partial $r=0.75$, $p<0.001$; male SLM, partial $r=-0.43$, $p<0.001$; multiple $r=0.88$, $n=46$). The two variables explained 77.4% of the total variance. Fifty-four per cent of the attempts in which the male had reached the female's genital pore with his snout ended with a contact between genitalia. This frequency was significantly correlated only with male SL (female SL, partial $r=0.07$, n.s.; male SL, partial $r=-0.36$, $p<0.03$; multiple $r=0.35$; $n=37$).

4. DISCUSSION

It is generally assumed that sneaky copulation in poeciliid fishes is a very inefficient mating tactic (Clark *et al.* 1954; Liley 1966; Farr 1980). This belief is based on the observation that the great majority of attempts are unsuccessful, overlooking that, under natural conditions, males of most poeciliids perform approximately one mating attempt per minute and thus several hundred attempts per day (Magurran & Seghers, 1994; Bisazza & Marin, 1995; Godin 1995). Our results suggest that insemination success through the sneaky tactic is not negligible, with an overall frequency of females inseminated ranging between 25 and 50% per day, and with a much higher success if one considers certain size classes (figure 1).

Our results indicate that a male mosquitofish's mating success decreases with its length, and increases with the size of the female pursued. As in a previous behavioural study (Bisazza & Marin 1995), the relative size of the male was the best predictor of male mating success. A similar finding was obtained using virgin females, thus excluding any possible influence on results deriving from the release of sperm from previous copulations. Large males might, however, be able to compensate for their lesser ability to obtain sneaky copulation by transferring more sperm. We did not find any significant relation between sperm number and male size. However, our estimation of sperm number within the female gonoduct was probably affected by a large measurement error and by the time elapsed since insemination, and our results on this topic are not conclusive.

The analysis of the behavioural observations suggested that the advantage of small males in sneaky copulations is due to two different factors. During the first part of a copulatory attempt, it is crucial for a male to get close to the female without being seen. Fifty-four per cent of all attempts abort at this early stage. The male must approach the female by swimming within the blind portion of the female's visual field. The extension of the blind portion of the visual field increases with female size, and small males should more easily approach a female while maintaining their body lined up behind her. Indeed, the probability of getting close to the female was positively correlated with female size, and negatively correlated with male size, with these two variables explaining about 77% of the variance in male success at getting near the female. A second important factor

is related to the ability of the male, at the end of a sneaky attempt, to rotate upwards and insert the tip of his gonopodium into the female's gonopore. At this stage, female size does not seem to play an important role, whereas male size is again negatively correlated with thrusting success. Since females cannot see males beneath them, the greater ability of smaller males to manoeuvre seems to determine this result.

Behavioural studies on five other species belonging to phylogenetically distant groups of poeciliids (Farr *et al.* 1986; Bisazza & Pilastro 1997) indicate that a small male advantage in sneaky copulation may be a general phenomenon in this family. This selective force may, in concert with other factors such as early maturation and reduced energy for locomotion (Zulian *et al.* 1993; Blanckenhorn *et al.* 1995), determine male dwarfism in many poeciliids. Indirect support for this interpretation comes from an interspecific survey showing that poeciliids which predominantly sneak-copulate tend to be more dimorphic in size than those in which the male achieves mating mainly by courting the female (Bisazza 1993). A small male advantage in sneaky copulation might also explain the coexistence, in some species, of genetically-determined small sneakers with large courting males (e.g. Gross 1985). Although the latter are usually favoured by both female choice and male-male competition, the sneaking strategy can also be very effective because female poeciliids are unresponsive to male courtship for a large part of their lifespan, and there may be many opportunities to pursue a female without having to compete with other males (Bisazza & Marin 1995). Conversely, large courting males must face intense competition for access to the few receptive females available at any time. Alternative mating strategies may therefore be maintained because of negative density-dependent selection or because the circumstances favourable to the two male types alternate in time or space.

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