

# Strong Reproductive Skew Among Males in the Multiply Mated Swordtail *Xiphophorus multilineatus* (Teleostei)

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## Abstract

Male swordtails in the genus *Xiphophorus* display a conspicuous ventral elongation of the caudal fin, the sword, which arose through sexual selection due to female preference. Females mate regularly and are able to store sperm for at least 6 months. If multiple mating is frequent, this would raise the intriguing question about the role of female choice and male-male competition in shaping the mating system of these fishes. Size-dependent alternate mating strategies occur in *Xiphophorus*; one such strategy is courtship with a sigmoid display by large dominant males, while the other is gonopodial thrusting, in which small subordinate males sneak copulations. Using microsatellite markers, we observed a frequency of multiple paternity in wild-caught *Xiphophorus multilineatus* in 28% of families analyzed, but the actual frequency of multiple mating suggested by the correction factor PrDM was 33%. The number of fathers contributing genetically to the brood ranged from one to three. Compared to other species in the family Poeciliidae, both frequency and degree of multiple paternity were low. Paternity was found to be highly skewed, with one male on average contributing more than 70% to the offspring. Hence in this *Xiphophorus* mating system, typically one male dominates and sneaker males do not appear to be particularly effective. Postcopulatory mechanisms, however, such as sperm competition, are also indicated by our data, using sex-linked phenotypes among the offspring.

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Swordtails of the genus *Xiphophorus*, live-bearing fishes in the family Poeciliidae, are excellent model organisms for studies of sexual selection because most species possess exaggerated male ornaments (Darwin 1871; Lambert and Lambert 1995). Specifically, a very conspicuous and colorful elongation of the lower fin rays of the caudal fin has evolved in these fishes, the so-called sword. Most commonly the sword has been viewed as a sexually selected trait, as *Xiphophorus* females prefer males with longer swords (Basolo 1990a,b, 1995). In the swordless platies, females also prefer males with artificially attached swords over conspecific males. This raises the possibility that the preference for a sword evolved because of a preexisting bias for swords in females of swordless species of *Xiphophorus* (Basolo 1990a,b, 1991). On the other hand, the brightly colored sword appears detrimental for survival and involves a higher risk of predation for males (Endler 1983; Rosenthal et al. 2001), which might be responsible for the inferred repeated loss of the sword within the genus *Xiphophorus* (Meyer et al. 1994). This is in agreement with the general notion that sexually selected characters involve significant costs (Wiens 2001).

A number of different hypotheses have been put forward to explain the evolution of costly male traits and their selective advantage by female choice. In resource-free mating systems (i.e., only sperm transferred during copulation), it is generally argued that female choice confers indirect benefits, such as the inheritance of “good genes” to the progeny (e.g., Reynolds and Gross 1992; Ryan 1991). In guppies, close relatives of the swordtails, multiple benefits for females have been indicated (Evans and Magurran 2000), including that males with the most developed sexually selected characters provide larger and/or better quality ejaculates to the female, thus leading to direct fertility benefits (Evans et al. 2003; Pilastro et al. 2002).

Male-male competition also plays an important role in the genus *Xiphophorus*, and often males use alternative mating strategies to achieve successful copulation with females. Most intriguingly, in *Xiphophorus multilineatus* (formerly known as the high-backed population of *Xiphophorus nigrensis*) the mating behavior of males is associated with a morphological phenotype and has a well-known genetic basis (Zimmerer and Kallman 1989). Four size classes can be

distinguished that differ in their Y-linked P alleles: small (s), intermediate-1 (I), intermediate-2 (II), and large (L) (see Zimmerer and Kallman [1989] for details). L-males have a long sword and their usual body length is above 40 mm, compared to s-males at the other end of the range, measuring only 25 mm on average with the sword being nearly absent (Lambert and Lambert 1995; Meyer et al. 1991). While males of the three largest size classes always engage in an elaborate courtship display, the small males show a range of different behaviors from frontal display to sneak-chase attempts (Zimmerer and Kallman 1989). The latter behavior has mostly been observed in the presence of large males, where small males resort to achieving copulation by gonopodial thrusts without cooperation from females. Thus mating competition in *X. multilineatus* occurs such that the L-males are dominant and deny the s-males access to the females.

Another important factor influencing the reproductive biology of all fishes of the family Poeciliidae, including *Xiphophorus*, is that females are able to store sperm for about 6 months (Turner and Snelson 1984; Van Oordt 1928; Winge 1937). Sperm is stored in folds of the ovary and gonoduct (Constanz 1989; Potter and Kramer 2000). Thus a male can fertilize several broods in a row with a single insemination, and multiple mating by females can lead to sperm storage, including the sperm of several males. Authors who genetically investigated the effects of multiple paternity in this family comprising the genera *Gambusia* (mosquitofish), *Heterandria* (killifish), *Poecilia* (guppy and molly), and *Xiphophorus* have reported a wide range of estimates (9–90% of females multiply inseminated) based on different genetic markers (sex-linked phenotypes in *Xiphophorus* [Borowski and Khouri 1976; Borowsky and Kallman 1976]; microsatellites in guppies [Kelly et al. 1999]; allozymes in mollies [Travis et al. 1990, Trexler et al. 1997]; microsatellites in *Gambusia* and *Heterandria* [Soucy and Travis 2003; Zane et al. 1999]). Since microsatellites are recommended for refined estimates of kinship and parentage (Queller et al. 1993), some of the studies using other marker systems (allozymes, male coloration) are expected to underestimate actual paternity frequencies.

Multiple mating is known to occur in a large number of animal groups (e.g., Ochando et al. [1996] for insects; Zane et al. [1999] for fishes; Hoekert et al. [2002] for reptiles; Burke and Bruford [1987] for birds), but whether there is a selective advantage either for females or their broods has long been puzzling for evolutionary biologists. Possible benefits of multiple mating by females include an increase in genetic diversity among the offspring and superior genetic quality (e.g., Birkhead and Parker 1991; Jennions and Petrie 2000). The incidence of sperm from different mates being simultaneously stored in the females' reproductive organs also generates the potential for sperm competition (Birkhead and Parker 1991) and cryptic female choice (Birkhead 1998). Nonetheless, the different contributions of male competition and female choice to total reproductive success are still poorly resolved. In this context it should prove highly insightful to examine the relative shares of paternity of preferred and nonpreferred males following multiple mating in species with female mate choice.

Most research on swordtails has focused on the mating behavior in captivity (e.g., Basolo 1995), which poses a number of difficulties with regard to applying the results to natural conditions. One possible way to avoid the problem of artificial environments in order to gain insights into mating success is to genetically dissect the number of fathers that contribute to the offspring. Here we employed polymorphic microsatellite markers to trace paternity patterns in the species *X. multilineatus* from wild-caught populations. Our goals were to determine both the frequency (proportion of broods sired by at least two males) and the degree of multiple mating (number of males genetically contributing to each brood) while completely relying on females caught in natural populations. Paternity variation among males contributing to multiply sired broods was also studied. We further attempted to correlate male offspring phenotype with the reproductive success of their putative fathers using sex-linked phenotypic markers.

## Materials and Methods

### Study Material

Samples of *X. multilineatus* Rauchenberger, Kallman and Morizot 1990 were obtained from the Rio Coy south of Ciudad Valles (San Luis Potosí, Mexico) at the point where the river is crossed by National Highway #85. The GPS coordinates are 21°45'01" N, 98°57'25" W. Fourteen pregnant females were caught and taken alive to Würzburg University in Germany. Another four females with young were killed immediately and the embryos extracted from the ovaries. Pregnant females were individually kept in small fish tanks at 23°C for several months in order to produce offspring. Females gave birth to one to four broods. After the young of each brood were born (usually no more than 10), they were separated from their mothers before they reached sexual maturity. Small fin clips about 1 mm<sup>2</sup> in size from mothers and their offspring were used for microsatellite genotyping.

Phenotypes of male offspring were recorded with regard to the size classes given by Zimmerer and Kallman (1989). However, we did not distinguish between the intermediate and large size classes since mating behavior between the two does not differ. The sex of some individuals could not be determined based on the morphology of the anal fin (e.g., Turner 1941) at the time the recordings were made. Thus we created a separate group of large individuals that could not be sexed. The genetic basis for the size polymorphism is well known, so that the father's size class could be inferred from the son because of a Y-linked locus controlling for size. For instance, s-males can only produce s-male offspring (Zimmerer and Kallman [1989] and the references therein).

### Microsatellite Genotyping

Genomic DNA was extracted from fin clips of single *X. multilineatus* using a high-salt extraction procedure according to Bruford et al. (1998). Seven microsatellite loci designed for

the closely related species *Xiphophorus montezumae*—*KonD6*, *KonD8*, *KonD21*, *KonD26*, *KonD29*, *KonT30*, and *KonT38*—were employed to assess paternity patterns within broods. Between 4 and 33 individuals were genotyped (mean 14.6, total 262) from each of 18 families, including mothers and their offspring. Primer sequences and general amplification conditions have been reported elsewhere (Seckinger et al. 2002). The forward primers were labeled with the fluorescent dyes TAMRA (*KonD6*), JOE (*KonD21*, *KonT38*), and 6-FAM (*KonD8*, *KonD26*, *KonD29*, *KonT30*). We multiplexed three to four different loci per lane on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Aliquots of 10  $\mu$ l polymerase chain reactions (PCRs) from all loci were combined in a final volume of 13–14  $\mu$ l containing 1  $\mu$ l of *KonD6*, *KonD8*, *KonD21*, *KonD26*, *KonD29*, *KonT30*, and *KonT38* PCR products. One microliter of this cocktail mixed with a size standard GS500 (Applied Biosystems) and high-performance liquid chromatography (HPLC) water were then denatured, electrophoresed, and visualized on the DNA sequencer. Alleles were scored using GeneScan 2.0.1 and Genotyper 3.7 (Applied Biosystems) software.

### Data Analysis

Paternal alleles were inferred by joint multilocus inspection for each family by comparing the progeny to their mother. Multiple paternity was concluded when more than two paternal alleles were found at a locus among the progeny of a family. Multilocus genotypes further permitted us to assign putative fathers in most cases, as certain allelic combinations from different loci of two putative fathers cannot co-occur in their respective offspring. For instance, when two paternal alleles in particular offspring at one locus were always found paired with two other paternal alleles at another locus, it was evident that these individuals were sired by the same male. Paternity by males was further confirmed statistically by showing nonsignificant departures from Mendelian expectations for the offspring of each inferred father using *G* tests for goodness-of-fit. On the basis of these assignments, we were able to compare paternity variation and its relationship to sex-linked morphological traits. *G* tests were used to examine the frequencies of different patriline within families under the null hypothesis that all males contributed equally to the offspring (Sokal and Rohlf 1995).

The effective number of alleles was calculated as  $n_e = 1 / \sum p_i^2$  (Hartl and Clark 1997). The program GENEPOP (Raymond and Rousset, 1995), via its Web implementation (version 3.1c), was used to test if the study populations conformed to Hardy-Weinberg expectations by examining the genotype frequency distributions of the wild-caught female samples. Genotypic linkage disequilibrium among all pairs of loci was investigated using Fisher's exact test as implemented in GENEPOP. *P* values were estimated by the Markov chain method with the parameters 1000 dememorization steps, 1000 batches, and 1000 iterations per batch for all tests. Bonferroni corrections were applied when multiple tests were performed (Sokal and Rohlf 1995).

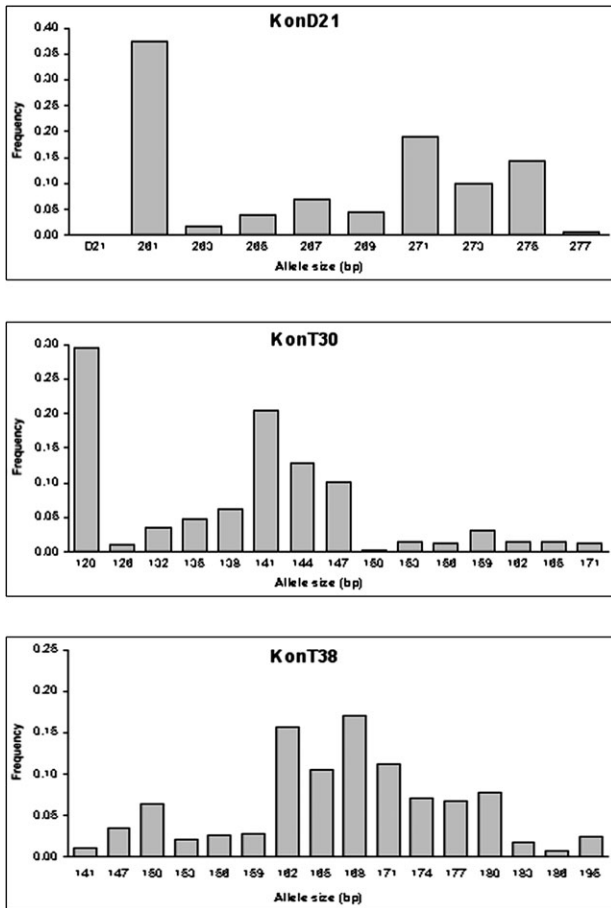
We used KINSHIP 1.3.1 (Goodnight and Queller 1999) in an attempt to distinguish full from half siblings by estimating likelihood ratios for the two hypotheses. Genetic relatedness among offspring of individual families was calculated using the RELATEDNESS 5.0.5 program (Goodnight 2000; Queller and Goodnight 1989). Allele frequencies were also estimated using Relatedness by weighting the frequencies within families equally. The unbiased effective number of fathers was estimated according to Pamilo (1993) as  $m_e = n - 1 / n \sum y_i^2 - 1$ , where  $y_i$  is the frequency of each father's offspring and  $n$  is the number of individuals analyzed. The probability of detecting multiple paternity from our microsatellite data was assessed using the PrDM software, which incorporates the effects of the number of loci, alleles, sample size, and genetic parents (Neff and Pitcher 2002). In addition, PrDM was used to adjust the observed frequency of multiple mating according to the following calculation: actual frequency = (observed frequency)/(PrDM).

To examine the variation of brood sizes and whether there were significant differences in mean brood size from the first to the last brood, we used a one-way analysis of variance (ANOVA) statistical test. *G* tests of independence allowing for multiple rows and columns (*R* × *C* test design) and applying William's correction were used (Sokal and Rohlf 1995) to determine whether, in the multiple paternity families, there was an association between the number of offspring sired per brood and the putative father. For instance, if one father was more frequently represented in the first two broods as compared to the last two broods, this would suggest the occurrence of incomplete sperm mixing or dwindling sperm stores of that male in the female.

## Results

### Population Estimates

A total of 53 alleles were found at seven microsatellite loci in a sample of 262 individuals including 18 females and 244 offspring. The most informative loci were *KonD21*, *KonT30*, and *KonT38*, as already pointed out by Seckinger et al. (2002) using fewer individuals. The size distribution and frequencies of alleles at these loci are shown in Figure 1. Ten, 15, and 16 alleles were observed at *KonD21*, *KonT30*, and *KonT38*, respectively. Alleles for the dinucleotide repeat *KonD21* always differed by 2 bp, while alleles for the two trinucleotide repeat loci *KonT30* and *KonT38* were separated by 3 bp (Figure 1), thus suggesting a stepwise mutation process. When looking at the effective number of alleles (Table 1), which is determined more by the uniformity of allele frequencies than by the actual number of alleles, the picture is similar in that locus *KonT38* is by far the most informative, with  $n_e = 9.86$ . According to Neff and Pitcher (2002), two loci with seven equally common alleles analyzed for 10 offspring would be required to detect multiply sired broods with 95% probability, which indicates that our three most polymorphic loci together should have enough power to detect most of the males that contributed to a brood.



**Figure 1.** Allele frequencies at the three most variable microsatellite loci—*KonD21*, *KonT30*, and *KonT38*—determined for 18 families of *X. multilineatus* by weighting the frequencies within families equally.

Genotype distributions in the population were within expectations of Hardy-Weinberg equilibrium at all loci (exact probability tests,  $P$  values invariably greater than .34). Maternal alleles segregated in all broods in accordance with the expected Mendelian proportions. Thus we found no evidence for the occurrence of null alleles at the microsatellite loci used. Among 21 individual tests for linkage disequilibrium between different loci, one remained significant (locus pair *KonD2*–*KonD29*;  $P = .001$ ) after adjustment of  $P$  values with Bonferroni methods. Genetic independence of these two microsatellite loci was therefore incomplete, but due to the low impact of locus *KonD29* ( $n_e = 1.77$ ), this is unlikely to have had a significant effect on the results.

### Multiple Paternity

Five of 18 families of *X. multilineatus* examined in this study were found to be sired by more than one male. In all offspring we detected 25 different males as putative fathers: 13 families with one father, 3 families with two fathers, and 2 families with three fathers. The frequency of multiple paternity among the broods of wild-caught females was

**Table 1.** Observed number of alleles ( $n_A$ ), effective number of alleles ( $n_e$ ), and expected heterozygosity ( $H_e$ ) for the microsatellite loci

	D6	D8	D21	D26	D29	T30	T38
$n_A$	3	2	10	4	5	15	16
$n_e$	1.42	1.36	4.61	1.96	1.77	6.07	9.86
$H_e$	0.30	0.26	0.78	0.49	0.44	0.84	0.90

therefore 27.8%. This result was mainly based on the straightforward criterion of the distribution of paternal alleles within families across loci, which also allowed us to assign putative fathers to their progeny. Kinship tests estimating likelihood ratios for full sibs versus half sibs were concordant, as the 13 singly sired families had for the most part highly significant results for being full sibs, while test results were incompatible with this hypothesis for the remaining 5 families. However, pairwise likelihood ratios in these latter families did not permit the unambiguous assignment of individuals to their putative fathers. No additional cases of multiple paternity among singly sired families were detected when testing paternal alleles for significant deviations from expected ratios of Mendelian inheritance, although some researchers have found the presence of previously undetected fathers using this approach (e.g., Travis et al. 1990; Zane et al. 1999).

Maternal and inferred paternal alleles for each of the multiple-paternity families—Xm3, Xm4, Xm7, Xm11, and Xm12—are given in Table 2. Among the offspring of families Xm3, Xm4, and Xm7, evidence for two fathers was strong because of support from at least two loci and reasonable proportions of progeny from the second father. In families Xm4 and Xm7, the contribution of a third father could only be detected at locus *KonT38*, which is the most polymorphic locus (Table 1). Families Xm11 and Xm12 had low offspring numbers and the second father was supported by one locus only. The single individual in family Xm11, for instance, might alternatively have resulted from a recent mutation event (locus *KonD21*, allele 277 to 275), as this is not an uncommon observation in microsatellites (e.g., Hoekert et al. 2002). However, Mendelian expectations for the offspring of each inferred father given in Table 2 were not significantly different from the observed numbers. Thus, according to our data, the degree of multiple mating in *X. multilineatus* lies between one and three.

### Paternity Variation

In the multiple paternity families of *X. multilineatus*, males did not contribute equally to the offspring, so that paternity shares varied considerably, as depicted in Figure 2. The general observation was the occurrence of a majority male, which produced the largest fraction of all offspring. The average percentage of offspring sired by this dominant male was 73.7% (range 66.7–80.0%), whereas second and third males' contributions to the offspring averaged only 15.7% (12.1–28.6%) and 6.6% (6.3–7.2%), respectively. Paternity variation was found to be statistically biased in three families

**Table 2.** Paternal contributions in multiply sired families of *X. multilineatus* at four microsatellite loci

Family	N	KonT30			KonT38									
		Mother	Father 1	Father 2	Mother	Father 1	Father 2	Father 3						
Xm3	25	144/160	141 (8)	144 (12)	144 (3)	150 (2)	142/183	149 (13)	156 (7)	153 (1)	168 (4)			
Xm4	14	120/147	120 (7)	140 (3)			146/174	156 (10)		162 (1)	165 (2)	180 (1)		
Xm7	33	135/153	120 (14)	147 (12)	120 (3)	141 (1)		162/171	162 (13)	177 (13)	149 (3)	177 (1)	168 (1)	174 (1)*
Xm11	6	120/138	144 (2)	147 (2)	144 (1)			149/171	165 (4)		165 (1)			
Xm12	7	120/156	132 (3)	141 (2)	132 (1)	141 (1)		162/168	146 (5)		168 (1)	174 (1)		

Note that the third father only showed up at locus *KonT38* and is therefore not shown at the other loci. The number of paternal alleles observed are shown in parentheses.

\* Individual cannot be assigned to this father with certainty.

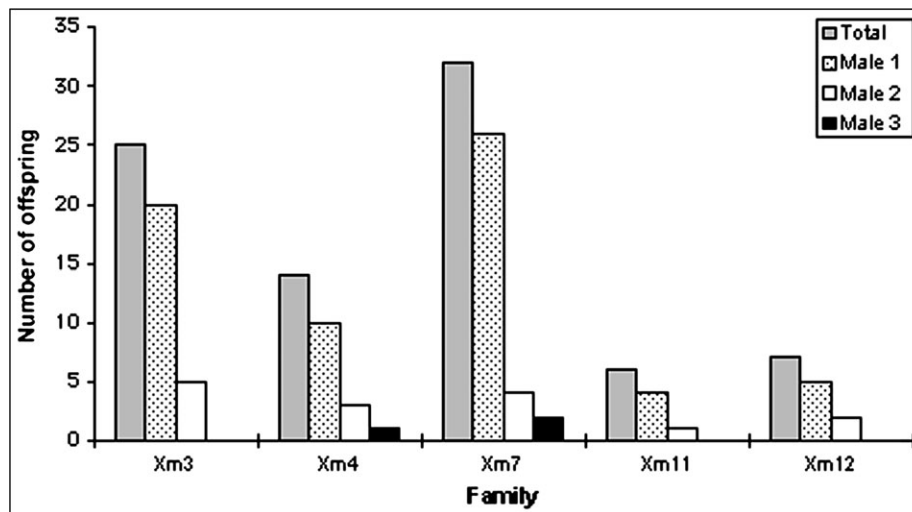
(Xm3,  $G_1 = 9.64, P = .002$ ; Xm4,  $G_2 = 9.28, P = .01$ ; Xm7,  $G_2 = 31.77, P < .0001$ ) where sample size was relatively large (mean 23.7). The other two families, Xm11 and Xm12, which had less than 10 offspring in total, were not significantly different from unbiased paternity probably due to small sample size.

The paternity frequency ( $\pm$  SD) across families was on average  $1.39 \pm 0.70$ . However, if paternity frequencies are highly biased, this value overestimates the genetic effects of multiple paternity. Calculating the effective paternity frequency is a useful measure that accounts for differences in paternity shares, which was mean  $m_e = 1.18 \pm 0.32$  for *X. multilineatus*. Genetic relatedness of family members was estimated to be  $r = 0.45 \pm 0.03$  (SE) in the whole population. This average relatedness estimate did not differ significantly from 0.5 (confidence interval [CI] = 0.39–0.51), which is the theoretically expected value in diploid organisms if only one male contributes to the offspring. Both effective paternity and genetic relatedness estimates suggest that multiple mating has a rather low impact on the genetic constitution of *X. multilineatus* when considering the population as a whole.

**Probability of Detecting Multiple Mating**

Using the allele frequency data for the whole population, we calculated probabilities of detecting multiple mating (PrDMs) in order to assess the accuracy of our paternity estimates. Different cases for two and three sires contributing to a brood were considered under varying paternity skews and offspring numbers (Table 3). To obtain a PrDM value of 99%, 10 offspring are sufficient if paternity among two males is unbiased. Because the paternity skew in *X. multilineatus* can be as high as 80%, one has to sample at least 15 offspring to have 95% confidence. If the skew is 90%, which appears not unlikely in *Xiphophorus*, up to 35 individuals are needed to confidently detect a second male. For the three-father families, while employing a skew setting close to the observed one (80%/15%/5%), we estimated nearly equivalent PrDM values (Table 3), suggesting a minimum sample size of 15 individuals.

Thus the analyses revealed we lacked power to detect multiple mating due to nonsampling rather than low marker variation. In 10 families we sampled fewer than 15 offspring, which considerably reduced the associated PrDM values (e.g., 70% for Xm11 with  $n = 6$ ). However, sample size was



**Figure 2.** Paternity variation in multiply sired broods (all successive broods combined) of *X. multilineatus*.

**Table 2.** Extended

KonD21					KonD29				
Mother	Father 1		Father 2		Mother	Father 1		Father 2	
263/273	275 (9)	273 (11)	273 (1)	275 (4)	286/286	286 (12)	288 (8)	286 (4)	
271/273	275 (5)	273 (4)	265 (1)	273 (2)	286/288	286 (10)			
263/277	273 (11)	277 (15)	275 (4)		286/286	286 (26)		284 (1)	288 (3)
263/263	277 (2)	269 (2)	275 (1)		286/286	286 (4)			
265/267	263 (1)	273 (4)	273 (2)		280/290	286 (5)		286 (2)	

inevitably limited to the small offspring number that females produce. The average PrDM across all families with the sample sizes used was 84.8% (range 31.8–99.6%, median 89.9%), indicating that the chance of nondetection was approximately 10–15% of multiply mated broods. Adjusting the observed frequency for PrDM, the actual frequency of multiple mating in our sample was 31–33%, depending on whether one uses the mean or the median.

### Brood Size

Since the first brood was separated from the mother before sexual maturity of the offspring, the sperm for the subsequent broods (usually up to three or four) must have come from the mother's sperm-storing organ. We investigated whether there was a difference in brood size from the first to the last brood, which may indicate possible sperm depletion over time. The weighted mean of all broods combined was  $5.9 \pm 3.0$  individuals per brood (range 1–11). However, there was no significant difference among brood sizes when comparing the means of successive broods across families (one-way ANOVA;  $F = 0.09$ ,  $P = .96$ ). We also recorded the number of embryos extracted from the ovaries of four pregnant females (mean  $7.8 \pm 4.3$ ) and compared offspring numbers with the first broods of live kept fish (mean  $6.3 \pm 1.7$ ). The 95% confidence intervals of both means did overlap, showing that there was no difference in the number of embryos produced and the actual number of living young that females released per brood under laboratory conditions.

We also examined the distribution of paternity among broods in four multiply sired families (data not shown). No

significant association between offspring number per brood and genetic father could be detected, that is, each of the two or three males' offspring were represented throughout the broods in a random fashion not deviating from the expected frequencies (Xm3,  $G_3 = 6.22$ ,  $P = .10$ ; Xm4,  $G_4 = 7.22$ ,  $P = .13$ ; Xm7,  $G_4 = 1.62$ ,  $P = .81$ ; Xm12,  $G = 2.25$ ,  $P = .52$ ). This indicates that available sperm from all mating partners are probably mixed in the female's sperm storage organ. The results further show that the sperm of a single male can last for up to four broods.

### Sex-Linked Phenotypes

Because we investigated offspring derived from wild-caught females, phenotypic information of males that had mated to these females was lacking. Nevertheless, we were able to infer phenotypes of fathers in 14 families on the basis of a Y-linked factor that shows no crossing over (Zimmerer and Kallman 1989), and classified whether male offspring fell into the large/intermediate or the small size class. Our results were consistent with the size at maturity in male offspring being determined by the inheritance of a Y chromosome from the father because all males attributed to a given sire, based on microsatellite data, also had the same phenotype.

Specifically, only one of nine families that were singly sired gave rise to small male offspring, while the other families exclusively produced large or intermediate males (Table 4). In the five multiple-paternity families, a more variable picture emerged. Families Xm3 and Xm7 produced only large males, although 2–3 fathers contributed to the offspring. Most interestingly, in family Xm4, three small male offspring were from the same father, whereas the other two fathers sired only large males. In Xm12, only small males from two different fathers were recorded, and Xm11 was uninformative because the sex of most offspring could not be determined.

**Table 3.** Estimated probabilities to detect multiple paternity in *X. multilineatus* under different paternity skews

Sample size	Probabilities (%) to detect multiple paternity				
	Two sires (%)		Three sires (%)		
	90/10	80/20	50/50	80/15/5	33.3/33.3/33.3
5	37.1	61.3	87.0	61.8	95.3
10	62.4	87.0	99.0	87.2	100.0
15	76.9	95.1	99.8	95.4	100.0
20	85.9	98.0	99.8	98.3	100.0
25	91.2	99.2	99.8	99.4	100.0
30	94.1	99.6	99.8	99.7	100.0
35	96.4	99.7	99.8	99.9	100.0

## Discussion

### Polyandry Within the Poeciliidae

In the microsatellite analysis presented in this study, we observed that 28% of broods in *X. multilineatus* were sired by more than one male. The actual frequency of multiple mating as suggested by the correction factor PrDM was 33%. When

**Table 4.** Sex-linked phenotypes recorded in 14 families of *X. multilineatus*

Family	Father	Number of individuals				Total
		L-male	s-male	Female	L-male/female	
Xm1	a	9	0	4	7	20
Xm2	a	3	0	0	4	7
Xm5	a	11	0	7	7	25
Xm6	a	3	0	0	12	15
Xm8	a	7	0	4	8	19
Xm9	a	7	0	4	5	16
Xm10	a	4	0	0	3	7
Xm13	a	1	0	0	8	9
Xm14	a	0	3	5	2	10
Xm3	a	7	0	7	8	22
	b	4	0	0	2	6
Xm11	a	1	0	0	3	4
	b	0	0	0	1	1
Xm12	a	0	1	0	1	2
	b	0	1	0	4	5
Xm4	a	3	0	2	5	10
	b	0	3	0	0	3
	c	1	0	0	0	1
Xm7	a	17	0	8	1	26
	b	1	0	3	0	4
	c	1	0	1	0	2

Females from which embryos were extracted are not shown ( $n = 3, 6, 9,$  and  $13$ ).

looking at patterns of multiple paternity within the genus *Xiphophorus* in a phylogenetic context (Meyer et al. 1994; Meyer A et al., unpublished results), it appears that the swordless platies, *Xiphophorus maculatus* and *Xiphophorus variatus*, show relatively high frequencies of multiple paternity (42–66%), even though color patterns, which have lower power than microsatellites, were used as a method of detection (Borowski and Khouri 1976; Borowsky and Kallman 1976). In contrast, the sword-carrying *X. multilineatus* has the lowest frequency of multiple paternity reported so far. Additional microsatellite data from small samples are in agreement with this, since multiply mated females were more frequent in the two platies mentioned above (60%,  $n = 5$ ) when compared with *X. nigrensis*, *Xiphophorus cortezi*, and *Xiphophorus birchmanni* (11%,  $n = 9$ ) as representatives of the northern swordtails (Seckinger J and Meyer A, unpublished results). Low rates of multiple paternity in swordtails may be related to strong sexual selection because female mate choice and male dominance limits the males' access to females. Similar effects of social behaviors on the mating system that allow single males to dominate are known from several groups of animals (e.g., Hutchings and Myers 1988; Jaeger et al. 2002). However, it is surprising that the molly (*Poecilia* sp.) can have very high frequencies of multiple paternity (up to 85%) despite showing similarities in mating strategies to the swordtails (Trexler et al. 1997).

Available evidence shows that multiple paternity is widespread in the Poeciliidae and the occurrence of species that mate only once has yet to be demonstrated. An

interesting observation, however, is that different populations can vary markedly in their mating system, with the consequence that paternity estimates are subject to high variance at the intraspecific level. For instance, in the sailfin molly (*Poecilia latipinna*), 9–85% of females showed multiple paternity associated with variation in female fertility (Trexler et al. 1997), and 25–64% multiply sired broods were recorded in guppies (Kelly et al. 1999). *Gambusia holbrooki*, a species that relies mainly on forced insemination attempts rather than on courtship, regularly has multiple-paternity frequencies greater than 90% (Zane et al. 1999). A recent study in *Heterandria formosa* suggests that higher population density leads to higher rates of multiple paternity; in this species 15–66% (mean 45%) of females were multiply inseminated (Soucy and Travis 2003). Our estimate for *X. multilineatus* falls below the population average for most other species of the family. Given the abundance in poeciliid fishes, multiple mating by females is likely to be an ancestral trait that was already present in the genus *Xiphophorus*. Furthermore, multiple paternity occurs in the sister genus *Priapella* (Seckinger J and Meyer A, unpublished results). In the future it will be necessary to study more species and map characters onto a well-established phylogeny.

### Reproductive Success of Males

Studies of paternity frequently reveal that mating behavior does not always reflect parentage. Considering that female *X. multilineatus* mate regularly with different males, at least under laboratory conditions, and have the ability to store sperm for more than 6 months, both the frequency and the degree of multiple matings (number of males contributing to each brood) was unexpectedly low. Likewise, there appears to be a high rate of mating in the mosquitofish (*Gambusia* sp.), yet genetics revealed only two to three sires per brood (Zane et al. 1999). Possible reasons for this could be that gonopodial thrusting need not indicate successful insemination or the existence of postinsemination processes (e.g., mechanisms of sperm competition or sperm utilization by females; for a more detailed discussion see below).

Polyandrous mating systems in which females typically copulate multiple times during each reproductive cycle, and yet show strong preference for certain male traits, are pivotal for understanding the mechanisms involved in sexual selection. In *X. multilineatus*, we found that the proportions of offspring sired by males differed considerably if females had mated with two or more males. Thus reproductive success was significantly biased, with the most successful male contributing on average more than 70% to the offspring. The following explanations for the occurrence of high paternity skew in natural populations of swordtails are discussed.

1. Precopulatory sexual selection. Alternative male strategies and female mate choice seem to be characteristic for many swordtail mating systems. In at least one other species, *Xiphophorus helleri*, males have been reported to be territorial and form social hierarchies where dominant

males try to drive subordinate males out of their home range (Franck et al. 1998). Dominant males are preferred by females and usually have longer swords and larger body sizes than subordinate males, indicating the importance of this trait in sexual selection. These behavioral factors are likely to account for the high number of singly mated females in *X. multilineatus*, which were probably monopolized by a dominant male. Dominant males of this species are always large or intermediate and deny the small males access to the females (Zimmerer and Kallman 1989). Our data concur with this observation, in that only one brood with few offspring was found to be completely sired by a small male. In most cases of multiple paternity broods, the reproductively most successful fathers were large or intermediate males.

However, subordinate males often manage to enter the territories of dominant males and try to inseminate females via gonopodial thrusting. This is a behavior by which males try to sneak inseminations by forced copulations, which has been reported for *X. helleri*, *X. cortezi*, and *Xiphoborus pygmaeus* (Franck 1964), and in *X. multilineatus*, is confined to the small male phenotype. In the laboratory, the reproductive output of small sneaker males was only 25% compared to large males (Zimmerer and Kallman 1989), showing that these forcibly achieved copulations might not be very effective. Since we don't have reliable data on the proportion of the small males in the sampled population, we can neither support nor refute the suggested low success of sneak-chase mating.

2. Postcopulatory sexual selection. We have to consider postmating mechanisms, such as sperm competition, as well as to be able to fully understand the incidence of high paternity skew in *X. multilineatus*. In spite of intense female bias in mating preference between L- and s-males, females also mated with more than one large male in two of the multiple-paternity families, yet paternity shares were highly skewed. There could have been different ejaculate sizes stored in the female with regard to mating speed (Pilastro et al. 2002), as well as poor sperm mixing (Harvey and Parker 2000), or the sperm of one male was qualitatively superior. Recent work in guppies showed that reproductive skew in males is greater than in females (Becher and Magurran 2004), and that colorful males achieve greater parentage than less ornamented ones (Evans et al. 2003). The latter observation can most easily be explained by the production of competitively superior ejaculates, which would reinforce the females' ability to choose.

Mating order also seems to play a crucial role in poeciliids, as some studies on sperm competition found a strong last-male advantage, suggesting that fresh sperm were at a competitive advantage over previously stored sperm (Hildemann and Wagner 1954; Winge 1937). In guppies, second-male precedence prevailed (with a few cases of first-male priority), even when two copulations took place within the same reproductive cycle, but a possible influence of female choice could not be excluded due to the

experimental design (Evans and Magurran 2001). Sperm choice by females following multiple mating has recently become a subject of growing interest (e.g., Birkhead 1998), and in general it could also account for paternity differences in live-bearing fishes. Nevertheless, current evidence for female sperm choice is limited, while the criteria used to provide conclusive evidence are still heavily debated (Birkhead 2000; Eberhard 2000; Pitnick and Brown 2000).

### Benefits of Multiple Mating

The advantage for males to mate multiply with different females is obvious, since they receive higher fitness through siring more offspring than if they only mated with a single female. The advantages for females to mate with several males are less clear (e.g., Birkhead and Parker 1991). Mating with more than one male increases the risk of infection and imposes higher predation risks (Magurran and Nowak 1991). But it has also been shown that females might profit from multiple matings with different males (reviewed by Jennions and Petrie 2000), mainly through genetic benefits of the increased genetic variability of multiply sired offspring, which is thought to increase survival rates, especially if environmental conditions are unstable. In guppies, laboratory offspring from multiply sired broods showed higher viability since they displayed better schooling abilities and escape responses than offspring from singly sired broods, but it remained unknown whether these behaviors were transmitted genetically (Evans and Magurran 2000). Moreover, Evans and Magurran found that multiply mated females had shorter gestation times and larger broods, and these multiple benefits might provide a reasonable explanation for the selective advantage of polyandry in some live-bearing fishes.

The impact of multiple mating on natural populations of *X. multilineatus*, however, is small, as shown by the population effective paternity being close to one. Hence a high genetic relatedness among individuals within a brood is retained, making them genetically more similar to singly mated populations. Given these circumstances, a selective advantage through genetic diversity benefits seems unlikely in species with little effect on their offspring's genetic variation. On the other hand, it is conceivable that sexual selection provides alternative benefits to the female by introducing strong paternity skew among males. If the sexually selected trait is a reliable guide to mating with genetically superior males, then females can increase both the quality and quantity of their offspring (for some evidence in guppies, see Evans et al. [2003]).

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