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Pronounced reproductive skew in a natural population of green swordtails, *Xiphophorus helleri*

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Abstract

For many species in nature, a sire's progeny may be distributed among a few or many dams. This poses logistical challenges — typically much greater across males than across females — for assessing means and variances in mating success (number of mates) and reproductive success (number of progeny). Here we overcome these difficulties by exhaustively analyzing a population of green swordtail fish (*Xiphophorus helleri*) for genetic paternity (and maternity) using a suite of highly polymorphic microsatellite loci. Genetic analyses of 1476 progeny from 69 pregnant females and 158 candidate sires revealed pronounced skews in male reproductive success both within and among broods. These skews were statistically significant, greater than in females, and correlated in males but not in females with mating success. We also compare the standardized variances in swordtail reproductive success to the few such available estimates for other taxa, notably several mammal species with varied mating systems and degrees of sexual dimorphism. The comparison showed that the opportunity for selection on male *X. helleri* is among the highest yet reported in fishes, and it is intermediate compared to estimates available for mammals. This study is one of a few exhaustive genetic assessments of joint-sex parentage in a natural fish population, and results are relevant to the operation of sexual selection in this sexually dimorphic, high-fecundity species.

Keywords: mating success, mating system, microsatellites, parentage analysis, reproductive success, sexual selection

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Introduction

Darwin (1871) introduced the concept of sexual selection in response to the conundrum posed by extravagant, typically gender-restricted, phenotypic features that seem to be of no adaptive value (and indeed may lower viability) in their bearers. Sexual selection on such heritable phenotypes normally plays out through male–male competition for mates (intrasexual selection) or via mating preferences by females (epigamic selection), and it requires that some individuals systematically leave more progeny than others due to differential mate acquisition. In theory, sexual selection is strongest when reproductive success varies widely among males (Clutton-Brock & Vincent 1991; Andersson 1994; Shuster & Wade 2003), which in turn is especially likely in polygynous species with a high variance in male mating success. A stronger correspondence of reproductive success with mating success in males than in females has long been regarded as a hallmark of intense sexual selection on males (Bateman 1948; Arnold 1994; but see Snyder & Gowaty 2007).

For many vertebrate species including mammals (e.g. Martin *et al*. 1992), birds (Birkhead & Møller 1992; Westneat & Stewart 2003), and fish (Avise *et al*. 2002), empirical descriptions of mating systems have benefited tremendously from molecular appraisals of genetic parentage (Avise 2004). However, assessing reproductive success in nature normally remains difficult, especially for males. Whereas the mean and variance in female mating and reproductive success can often be estimated by counting eggs or offspring from each dam in a large sample, estimating these same reproductive parameters in males is

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far more challenging because a sire's progeny may be distributed among several or many dams. To date, genetic appraisals of male mating success and reproductive success have been limited mostly to species with reliable pedigree or long-term census data, such as several mammals (Say *et al*. 2003; Hayes *et al*. 2006; Rossiter *et al*. 2006; Vanpé *et al*. 2008), birds (Alatalo *et al*. 1996; Reynolds *et al*. 2007), and lizards (Lebas 2001; Morrison *et al*. 2002). Even in such favourable logistical circumstances, however, substantial numbers of progeny often cannot be ascribed to known sires. Successful attempts at complete paternity assessment in other animal groups are few (Jones *et al*. 2002; Gopurenko *et al*. 2007).

Some early attempts to genetically evaluate the number of females that mate with particular males were performed on fish species in which males guard nests (Rico *et al*. 1992). In such cases, reproductive output and mate numbers can be estimated for focal (nest-tending) males, but the settings are not conducive to obtaining full information on males who may be involved in alternative reproductive tactics and produce progeny dispersed among multiple nests (Neff 2001; Fiumera *et al*. 2002). Another favourable situation in nature is presented by the Syngnathidae (pipefishes and seahorses), in which the pregnant males carry 'packaged' progeny that permit evaluations of male reproductive success (Jones & Avise 2001). In addition to these special cases, a few successful attempts have been made to estimate overall male reproductive success of fish in closed artificial systems (Becher & Magurran 2004; Spence *et al*. 2006; Reichard *et al*. 2008) or in relatively closed natural populations that can be nearly exhaustively sampled (Gross & Kapuscinski 1997; Blanchfield *et al*. 2003). Some of the most detailed such studies of mating and reproductive success and their skews in both male and female fishes have been conducted on anadromous salmonids that return to particular creeks to reproduce (Garant *et al*. 2001; Araki *et al*. 2007, 2008).

The green swordtail (*Xiphophorus helleri*; Poeciliidae) provides an excellent opportunity to investigate female and male reproductive success because (i) females give birth to live young, which facilitates collecting complete broods, and (ii) swordtails inhabit areas in creeks that often become isolated from one another during the dry season, which allows exhaustive sampling due to the limited size and connectedness of such sites. At our study site (Firetail Creek, a tributary to the Bladen Branch River draining the Maya Mountains in Belize), water flow is continuous during the wet season, but during the dry season most shallow stretches between deeper pools dry out. This produces a string of discrete or quasi-discreet pools that we assume are isolated sites of closed habitat during the dry season. A complication is that females are able to store sperm for months (Constantz 1989), but our sampling design (see below) takes this into account by sampling focal and adjacent pools multiple times. This system therefore offers a special opportunity to study reproductive success and skew because each pool has a limited but nonetheless adequate number of swordtails that can be sampled almost exhaustively for parentage analysis.

The genus *Xiphophorus* is a model system in sexual selection. Males establish dominance hierarchies and compete for access to females whereas females are choosy and obtain nothing but sperm from males (Franck & Ribowski 1993; Meyer 2006). Male swordtails exhibit a conspicuous, sword-like appendage of the caudal fin, despite substantial costs (Rosenthal & Evans 1998; Rosenthal *et al*. 2001; Basolo & Alcaraz 2003), and females prefer males with longer swords (Basolo 1990a). The species *X. helleri*, in particular, furnishes a textbook example of the pre-existing bias theory illustrating that the sword evolved in response to a pre-existing bias of females (Basolo 1990b).

Genetic analyses in a related species (*Xiphophorus multilineatus*) uncovered that broods obtained from wild-caught females were fathered by up to three males (Luo *et al*. 2005), and also documented paternity skew in multisire families, with the most successful males fathering on average more than 70% of the offspring within a brood. However, Luo *et al*. (2005) did not attempt to exhaustively sample females and because no males were collected, the minimum number of sires could only be estimated per brood. By design, these estimates of paternal reproductive skew within a brood leave unanswered the question most relevant to sexual selection: What is the male reproductive skew when a study also takes into account males who mate with multiple females, and males who fail to sire any offspring?

Here we use definitive molecular markers of genetic parentage to measure and compare the means and variances of both mating success and reproductive success of male (and female) green swordtail fish in an exhaustively sampled natural population. Specifically, we address the following: What is the mating system in this population? Do a few males monopolize the matings or is breeding equitably distributed? And is mating success (the number of females carrying at least some of a sire's offspring) strongly correlated with reproductive success (the total number of offspring sired by a male)?

Materials and methods

Study system and material

Our goal was to exhaustively sample pregnant females as well as the potential sires of their broods at discreet sites in Firetail Creek. It would be insufficient to collect only males currently present at the sites as potential sires because female *Xiphophorus helleri* can store viable sperm for months and males in adjacent pools, or those that were present at a site but later died, are also potential sires. We therefore

sampled males multiple times and in several adjacent pools in both upstream and downstream directions from our focal pools. Specifically, we sampled males throughout the lower length (about 1 km) of Firetail Creek at the beginning (December 2006) and near the end (April 2007) of the dry season. Males were caught once more in the two focal pools (see below), where females were collected, during the final collection in May 2007. Males were brought to a field laboratory, anesthetized with MS-222, photographed, measured, sampled for a small (1×2 mm) piece of caudal fin preserved in dimethyl sulphoxide solution (Seutin *et al*. 1991), and individually marked by injecting small amounts of biocompatible, coloured elastomer (North-west Marine Technology Inc.). Males were released the next day at their site of origin.

In May 2007, adult females were collected from two pools in Firetail Creek (FT3/4 and FT5; global positioning system coordinates in universal transverse Mercator format: FT3/4 = 16Q 0316244 1831904 and FT5 = 16Q 0316167 1831925). Pools were about 65 m apart and separated by dry gravel and stretches of shallow surface flow. These pools were chosen because their physical characteristics facilitated fish collection, and because the number of fish was sufficient for meaningful comparisons of reproductive parameters yet small enough to make genetic screening feasible for all progeny. Females were sampled exhaustively at FT3/4: we spent approximately 50 person hours over 2 days sampling FT3/4 and caught no adults in the latter part of the second day. We continued to check FT3/4 for about six person hours on two subsequent days and again neither observed nor caught adults. At the second site, FT5, we estimate to have sampled over 95% of females during 60 person hours (but the sampling was not exhaustive because two females were seen at a later time).

Females were transported live to a laboratory at University of California, Los Angeles and kept in individual aquaria until birth of the first brood. We recorded standard length after females arrived in the laboratory and again after giving birth. Tissue samples of the females and the progeny were preserved in dimethyl sulphoxide solution for genetic analysis. Four females died before giving birth (two females per pool) and two others had not produce progeny after 9 months. The remaining 69 females produced 1476 offspring, all of which were genotyped at nine polymorphic loci. Additionally, we genotyped a second brood ($n = 17$) from one female, but we include these data only in the calculations of *de novo* mutation rates.

The impact of removing females on Firetail Creek population should be minimal because: (i) swordtails mature on a continuous basis and we did not remove juveniles; (ii) pools become reconnected during wet season allowing recolonization from neighboring sites; (iii) we took only a small sample relative to the whole population in Firetail Creek, which has about 15 pools in the lower 1-km section; (iv) some pools dry out naturally during the dry season, so that extinction and subsequent recolonization of a pool are parts of a natural cycle in this system.

Microsatellite genotyping

Genomic DNA was extracted from fin clips of adults or from posterior body parts of fry using proteinase *K* tissue digestion followed by phenol–chloroform–isoamyl extraction and ethanol precipitation (Milligan 1998).

We chose genetic markers for our study from a database of microsatellite loci developed for *Xiphophorus* (Walter *et al*. 2004). Initially, we selected 13 loci using the criteria that the loci should: (i) encompass tri- or tetranucleotide repeats (to facilitate reliable scoring), and (ii) not belong to the same linkage groups (to ensure independent transmission). We then evaluated variation levels on agarose gels using polymerase chain reaction (PCR) products from 28 *X. helleri* adult individuals. Four loci were rejected at this stage because they displayed heterozygosity levels less than 20%.

For the remaining nine loci, one primer in each pair was labelled with a fluorescent dye (HEX, 6-FAM, or NED) and amplified in four PCR sets: set 1, Msb080, Msd045, Msb069; set 2, Msd033, Msd036, Msd051; set 3, Msd060, Msd055; and set 4, Msc045. Locus Msc045 co-amplified poorly when multiplexed with other loci. Therefore, Msc045 was amplified separately and then pooled with Msd060 and Msd055 before electrophoresis on an ABI 3130xl automatic sequencer. The PCR cocktail (final volume 10 μL) for the co-amplified loci consisted of 1× Go*Taq* reaction buffer (which included 1.5 mM $MgCl_2$), 0.25 μg bovine serum albumin, 0.2 mM each dNTP, 0.25 μM each primer, 0.4 U Go*Taq* DNA polymerase (Promega), and 1 μL genomic DNA. Amplification of Msc045 was carried out under similar conditions except that 0.25 U of Go*Taq* polymerase were used.

Amplifications were conducted under an initial denaturation step at 95 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 40 s, annealing at 51 °C for 40 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. Multiplexed PCR products were diluted 14- to 20-fold, after which 1 μL of diluted product was mixed with 9.6 μL of deionized formamide and 0.4 μL size standard GS500 (ROX labelled; Applied Biosystems), denatured for 4 min at 95 °C, and electrophoresed on an ABI 3130xl. Alleles were scored using GeneMapper 4.0 (Applied Biosystems).

To evaluate scoring error, we did secondary scoring for 117 individuals at all nine loci and obtained identical genotypes for all. Genotyping efficiency was high; all adults and 1490 progeny were genotyped at all nine loci. Three offspring were genotyped at eight loci; they were inferred to be homozygous for null allele at one locus (see Results).

Paternity assignment and data analysis

For the paternity assignments, a strict exclusion approach usually enabled us to specify the sire (see Results). In most of the relatively few cases where this was not feasible, it was necessary to assume the presence — typically at one locus only — of a new mutation or a null allele in an otherwise compatible multilocus paternal genotype. Similar multilocus genetic comparisons of progeny with their known mothers (the identities of which were beyond doubt from the direct evidence of pregnancy) supported the interpretation that occasional null alleles and *de novo* mutations were indeed present in this population (see Results). Alleles (in offspring) inferred to be due to *de novo* mutations differed in length by at least 3 bp from their original state in a parent, and thus were unlikely to be a result of scoring errors.

To help identify sires (paternity), we used the program Cervus 3.0 (Marshall *et al*. 1998; Kalinowski *et al*. 2007), which adopts a maximum likelihood approach that takes into account estimated allele frequencies in the population. The program calculates a logarithm of odds (LOD) score (the logarithm of the likelihood ratio of parentage of a particular candidate parent), and, using simulations, it determines the confidence of assignment of the most likely candidate parent(s). The program also shows mismatches (if any) between parents and progeny, and indicates when these mismatches could be due to null allele. Exclusion probabilities were calculated according to Jamieson & Taylor (1997) and Waits *et al*. (2001) as implemented in Cervus.

Initial runs of Cervus under a variety of parameters showed that the candidate sires invariably remained the same (despite some variation in LOD scores among runs). The following parameters were used for the simulations for values reported in Results: proportion loci typed = 0.999, proportion loci mistyped = 0.001, proportion sires sampled = 0.90, simulated genotypes = 50 000. All assignments of paternity reported from Cervus were carried out at the strict confidence level of 95%.

The exclusionary approach alone could not select between two candidate fathers for four offspring (from four broods). In these cases, we chose males with highest LOD score (assignment with 95% confidence). Further evidence that these were true fathers came from the observation that the selected males sired between 8 and 62 other offspring in these broods, whereas other candidate fathers did not match any other offspring.

The standard presence of different sets of full-sib cohorts within multiple-sired broods facilitated visual inspections of the data and helped to confirm the reconstructions of paternal genotypes for males who were not sampled (see Results).

We compared general shapes of the distributions of the number of mates and number of offspring between males and females using the nonparametric Kolmogorov–Smirnov two-sample test (Sokal & Rohlf 1995, pp. 434–439). Association of the number of mates and number of offspring was tested with Spearman's coefficient r_S (Sokal & Rohlf 1995, pp. 598–600). Correction for multiple testing was carried out using the sequential Bonferroni method (Sokal & Rohlf 1995, p. 240). Probabilities from independent tests of significance were combined using Fisher's method (Sokal & Rohlf 1995, pp. 794–797). Departures from Hardy–Weinberg equilibrium were assessed separately for each locus using exact tests as implemented in GenePop (Raymond & Rousset 1995). Tests for null alleles were further conducted with software Micro-Checker (van Oosterhout *et al*. 2004).

The degrees of mating and reproductive skew were measured with a binomial skew index B (Nonacs 2000) that can range from minus one to plus two. B calculates the observed variance in reproductive skew corrected by the expected variance if all individuals were stochastically equal in success. A value of zero indicates a random distribution of offspring or mates among parents; positive values indicate skew; and significant negative values suggest an overly even distribution of offspring or mates. Significance levels were estimated by simulation with 10 000 permutations, and were nearly identical in repeated runs.

Results

Population variation and the certainty of paternity assignments

Molecular features of the nine loci employed in the current study are summarized in Table 1. A total of 162 different alleles were detected in our sample of 227 adults (69 females and 158 males) from lower Firetail Creek. Mean heterozygosity was 0.84, and the mean number of alleles per locus was 18. This high level of genetic variation makes these molecular markers extremely powerful for parentage assessments. The parent-pair non-exclusion probability was 2.3×10^{-10}. Pairs of unrelated individuals were unlikely (4.5×10^{-15}) to share a multilocus genotype, and even pairs of full siblings had a low expected mean probability (3.6×10^{-5}) of genetic identity across all nine loci. Indeed, empirically, no two individuals in our entire study (including full siblings) were identical across all surveyed microsatellite loci.

Two loci (Msd045 and Msd060) significantly departed from Hardy–Weinberg equilibrium (HWE) in our adult population sample due to heterozygote deficiency. Micro-Checker (van Oosterhout *et al*. 2004) suggested the presence of null alleles at these loci in the sample of adults. These were the same loci at which some progeny in several broods appeared to lack a parental allele due to the presence of nulls (see below), which nevertheless did not unduly complicate the parentage assignments.

Table 1 Characterization of nine microsatellite loci in green swordtails, *Xiphophorus helleri*

Locus	Repeat motif	Primer sequences	N_a (size range, bp)	H_O	H_E
Msb069	(ATG)	F: GATCTGTCAGCCATGTCCAGAAG; R: NED-TGGTCACATAGTAACCTACGGGTC;	12 (111–165)	0.833	0.855
Msb080	(ATG)	F: FAM-TTGTGGATGCTACAGAATCAGACA; R: TCTATTAAAGTGGACTGAACAGGGC;	13 (100–139)	0.824	0.843
Msd045	(TAGA)	F: HEX-CCCCGTAATAATCTGTTACCCCA; R: CCCTTTAAAACCTCTTTGACTTCCCTT;	16 (124–184)	0.793	0.915
Msc045	(TACA)	F: TACGTGTCCAGTTAAACCAAAAAAGTAT; R: NED-TCTGCAAAAGTCATGTTATCAAAACA;	12 (177–237)	0.775	0.774
Msd055	(TAGA)	F: FAM-TGGTGCTGCGTGGAAGATT; R: TTAGACTCTACTGCTCAGACACTGCA;	22 (164–256)	0.903	0.892
Msd060	(TAGA)	F: GATCTCAGTTTAACCACAACAGGGT; R: HEX-CCCTGCTGGTTCGTCTGG;	15 (134–202)	0.780	0.890
Msd033	Irregular	F: GGGATTAGTGGCTGTTATTAATGGG; R:FAM-TTGGACGAGTAAGAAGAGTAATCAAATT;	39 (207–349)	0.930	0.935
Msd036	(TAGA)	F: GTGGTGTAGATCGTGTTCCTTTGTA; R: HEX-TGCACGTGAATCAGAAGGCTCTT;	14 (137–195)	0.872	0.900
Msd051	(TAGA)	F: GCATCCCCACAGTATAATTCTGCT; R: NED-CACGTGGTTTGAAAATGTCGAA;	19 (162–230)	0.885	0.902

N_a, number of different alleles observed in 227 adults; H_O, observed heterozygosity; H_E, expected heterozygosity.

A total of 142 of the 1493 offspring (nearly 10%) appeared to 'mismatch' their known mothers at one locus, and an additional 15 offspring (1%) mismatched their respective dams at two loci. Among the single-locus mismatches, for 129 progeny these were due to null alleles, and for the other 13 progeny these were attributable to *de novo* mutations. The 15 instances of two-locus mismatches were caused either by the presence of a null allele at one locus and a *de novo* mutation at another (two such cases), or by null alleles at two loci (13 such progeny, all within one brood whose dam was heterozygous for null alleles at both Msd045 and Msd060).

Most of the offspring (1364 individuals, or 91%) matched, at all nine microsatellite loci, a candidate sire that was included in our collection. Among the remaining 129 progeny, 65 specimens (about 50%) showed mismatches from all candidate sires at one or two loci, apparently due either to null alleles or *de novo* mutations. Nearly all such mismatches were at one locus; only two progeny had two-locus mismatches (apparently due to their sire being heterozygous for null alleles at two loci). Most of the single-locus mismatches could be attributed to null alleles, but in 13 cases, they were apparently due to newly derived mutations. Despite these occasional mismatches, all offspring were assigned paternity at the 95% confidence level or higher.

For the other 64 progeny with allelic mismatches to all adult males collected, the number of mismatches per individual ranged from two to six (mode and median = 4). These offspring — apparently sired by unsampled males — were distributed among nine broods. Each such ensemble of mismatched progeny within a brood collectively carried no more than two alleles per locus, suggesting one sire per ensemble. For four such ensembles (with 56 progeny in total), we were able to reconstruct the multilocus genotype of the presumptive sire. When compared among themselves, pairs of these multilocus genotypes were found to be identical suggesting that two males each had mated with two females (the fact that the alleles segregated in accord with Mendelian expectations further supported this inference). The other eight progeny in five broods must have been sired by a total of at least four males (whose multilocus genotypes could not be reconstructed completely due to small progeny number).

Thus, among the total of 1493 offspring from the 69 females, 1429 (96%) could be assigned to specific males that we had collected and genotyped. Fifty-six of the remaining 64 progeny (in four broods) could be assigned to either of two males whose genotypes we reconstructed at all nine loci, and the other eight progeny (from five broods) could have been sired by any of at least four males. In total, we genetically documented 50 different sires (44 of which had been captured and assayed, and six of which had not been captured but whose paternity was deduced).

De novo *mutations*

We observed 33 cases of parent–offspring genotypic inconsistency that appear to register *de novo* mutations.

Twenty-eight of these were independent singletons (mutations each confined to one progeny), and one was a clustered mutation (Jones *et al*. 1999) carried in this case by five (among 98) offspring sired by male FTc04. Interestingly, copies of this clustered mutation, which apparently arose in FTc04's germline and were distributed to multiple successful gametes, were found in broods from three different females with whom FTc04 had mated. Overall, 15 of the independent *de novo* mutations were of maternal origin, nine arose in males, and five could have arisen with equal probability on either the maternal or the paternal side.

Because we screened in effect a total of 13 437 gametes (1493 progeny × 9 loci), the estimated total incidence rate of new mutations in progeny is 2.5×10^{-3}, and the rate of origin of *de novo* independent mutations in the germline of parents is 2.2×10^{-3}. These estimates are within the range reported for microsatellite loci in other species (Goldstein & Schlötterer 1999). Nearly 50% of the independent mutations (14 out of 29) occurred at the only locus in our study (Msd033) characterized by an irregular repeat motif (Table 1).

Null alleles

As deduced from the genotypic composition of broods, four among the 69 adult dams in the current study were heterozygous for a null allele at either Msd045 or Msd060, and one was simultaneously heterozygous for null alleles at both loci. The frequencies of null alleles in adult females were thus 0.036 at both Msd045 and Msd060. These null alleles segregated in all broods in Mendelian fashion. Null alleles in males were likewise restricted to loci Msd045 and Msd060. Among the 46 sires (excluding four uncollected specimens whose genotypes could not be fully reconstructed), three were deduced from progeny analyses to be heterozygous for a null allele at Msd060, one was heterozygous for a null allele at Msd045, and one was heterozygous for a null allele at both loci. Thus, the estimated frequencies of null alleles in adult males are 0.043 and 0.022 at Msd060 and Msd045. These null alleles segregated in expected Mendelian ratios within each brood.

For both sexes combined, null allele frequencies at Msd060 and Msd045, as deduced directly from these parentage analyses, were 0.039 and 0.030. These values are similar to a null-allele frequency estimate of 0.040 (from Cervus) based on the observed magnitude of population-level departures from HWE at each of these two loci.

Mating success

The 27 adult females from site FT3/4 produced a total of 627 offspring. The mean number (± SD) of progeny per brood was 23.3 ± 15.4 (range 4–63). These offspring were genetically assigned to 20 sires, 11 of which were local (i.e. captured at the FT3/4 site at least once). As deduced from the progeny analyses, at least 48 successful mating events had taken place at that location. Most of the offspring (461, or 73%) were sired by local males, who also accounted for most of the successful matings (36, or 75%) at this site. The 42 adult females from the FT5 location produced a total of 849 offspring via 35 sires. The mean number of progeny per brood was 20.2 ± 14.0 (range 3–57). At least 74 mating events were necessary to explain paternity in these broods. Most of the offspring (679, or 80%) were sired by 19 local males, who also accounted for most of the successful matings (54, or 73%) at this site.

The mean number of fathers per brood (i.e. successful mates per female) was 1.8 ± 0.7 at both FT3/4 and FT5. Table 2 dissects these patterns by showing numbers and percentages of females who had mated successfully with specified numbers of males. Forty-four of the 69 broods (64%) were deduced to have been sired by at least two males, and about 11% of broods were sired by three or four specifiable males. There was no statistical difference between sites FT3/4 and FT5 in the mating patterns by females ($\chi^2 = 1.89$, d.f. = 3, $P = 0.60$).

Males often had multiple mates also, as deduced from the paternity analyses of all examined broods. As detailed in Table 3, successful males on average had about 2.2 mates each, with local long-term resident males typically faring best (averaging as many as 5.4 mates each at site FT3/4).

Table 2 Successful mating events by female *Xiphophorus helleri* as deduced from paternity analyses of individual broods. Shown are numbers (and percentages in parentheses) of females whose broods had the indicated numbers of sires

Number of mates	Site FT3/4	Site FT5	Total
1	10 (37.0%)	15 (35.7%)	25 (36.2%)
2	14 (51.9%)	22 (52.4%)	36 (52.2%)
3	2 (7.4%)	5 (11.9%)	7 (10.1%)
4	1 (3.7%)	0	1 (1.4%)
Total	27	42	69

Table 3 Mating success of male *Xiphophorus helleri* as deduced from paternity analyses of all broods. Shown are the mean numbers (± SD) of mates per sire

Category	Site FT3/4	Site FT5
All males that sired progeny	2.4 ± 1.9	2.1 ± 2.2
Local successful males*	3.3 ± 2.2	2.8 ± 2.8
All local males†	2.0 ± 2.4	2.4 ± 2.7
Long-term residents§	5.4 ± 1.3	4.4 ± 3.9

*Adult males who sired progeny and who were caught at the site at least once; †adult males (including those who did not produce progeny) who were caught at the site at least once; §adult males who were caught at the site in both 2006 and 2007.

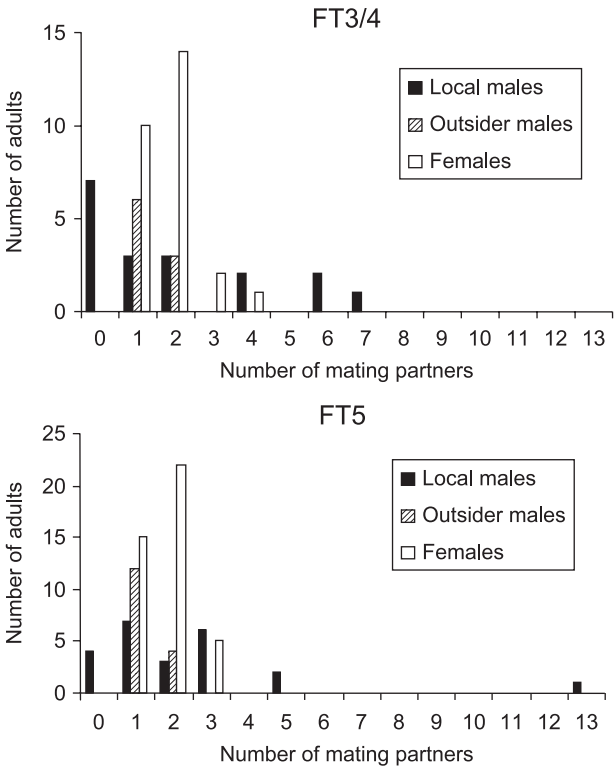

Fig. 1 Numbers of mating partners for individual females (open bars) and males (closed and hatched bars) of *Xiphophorus helleri*. For the males, mate counts are shown separately for 'local' sires (those captured in the same location) and males caught elsewhere in the stream.

Male and female mating success (mate numbers) are also summarized in Fig. 1. There were no significant differences between males and females in the overall distribution of partner numbers at either FT3/4 ($D = 0.21$, $n_1 = 27$, $n_2 = 20$, $P = 0.62$) or FT5 ($D = 0.22$, $n_1 = 42$, $n_2 = 35$, $P = 0.28$), according to Kolmogorov–Smirnov test. The skew index (B) in mating success did not differ between males and females at FT3/4 ($P > 0.05$), nor was the observed skew in either sex significantly different from the expected B value of zero under random mating (males, $B = 0.011$, $P = 0.051$; females, $B = –0.014$, $P = 0.99$). However, mating skew for males was significantly higher than for females at FT5 (males, $B = 0.017$; females, $B = – 0.01$, $P < 0.05$), and was also significantly higher for males than expected under random mating ($P = 0.003$).

Reproductive success

Figure 2 plots the distributions of paternity within each of the 69 broods. Among the 44 multiple-sire broods, progeny contributions by different fathers were significantly unequal ($P < 0.01$ in χ^2 tests for independence) in 29 cases (66%), and 21 tests remained significant ($P < 0.05$) after sequential Bonferroni correction. Fisher's test using combined probabilities was also highly significant ($\chi^2 = 711.3$, d.f. = 88, $P < 0.001$). Furthermore, the test of combined probabilities was also significant ($\chi^2 = 30.9$, d.f. = 16, $P < 0.01$) when applied to broods with more than 10 offspring each but that had not shown significant departures from equality of paternity in the individual χ^2 tests. The average proportion of offspring sired by the most successful male in a brood was 80% (range 43–98% among broods). The next successful male sired on average 18% of a brood (range 2–50%), and the third and fourth most successful males sired on average 11% (3–27%) and 4% (one brood only) of the progeny. Thus, an overall tendency for biased contributions by sires to broods is well confirmed.

As shown in the upper half of Fig. 3, the number of sires per brood was not significantly correlated with brood size at either collection site (at FT3/4, Spearman's coefficient, $r_S = 0.18$, $n = 27$, $P = 0.36$; at FT5, $r_S = 0.11$, $n = 42$, $P = 0.49$).

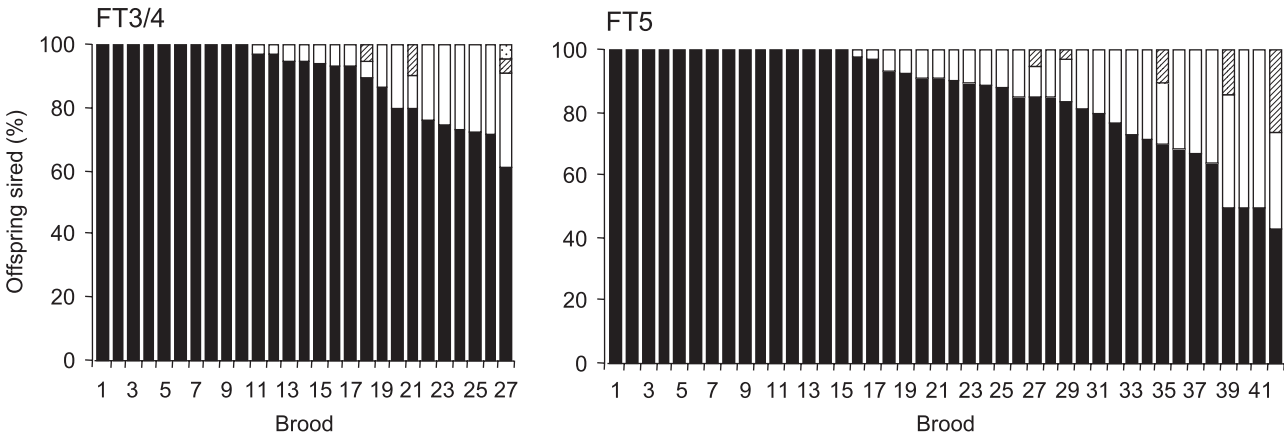

Fig. 2 Percentages of offspring from various sires within each of 69 broods in FT3/4 and FT5 sites. Each type of shading indicates a different sire (but does not imply that sires in different broods were the same).

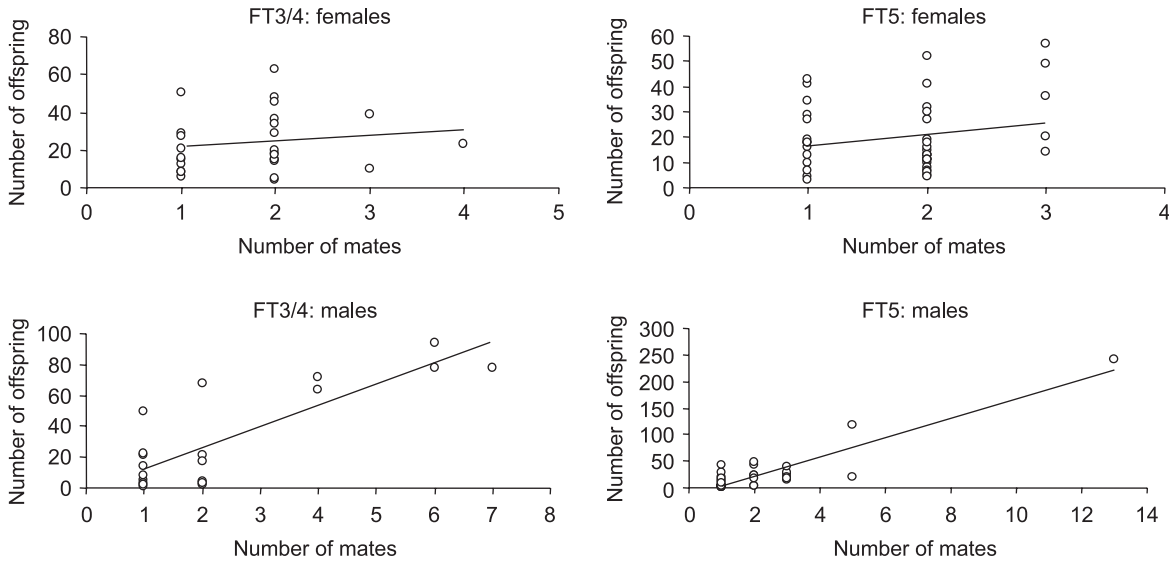

Fig. 3 Relationship between mating success and reproductive success in females (top) and males (bottom).

In other words, there was no evidence that a female's reproductive output was related to her mating success (number of males whom she mated). By contrast, male reproductive output was strongly correlated with number of female mates (lower half of Fig. 3): at FT3/4, $r_S = 0.62$, $n = 20$, $P = 0.003$; and at FT5, $r_S = 0.62$, $n = 35$, $P < 0.001$.

Overall, the average numbers of offspring per successful male were 31.4 ± 32.2 and 24.3 ± 43.8 at FT3/4 and FT5, respectively. The distribution of offspring numbers did not differ among successful males and females at either FT3/4 (Kolmogorov–Smirnov two-sample test, $D = 0.30$, $n_1 = 27$, $n_2 = 20$, $P = 0.21$) or at FT5 ($D = 0.30$, $n_1 = 42$, $n_2 = 35$, $P = 0.1$). For each sex at each site, the skew in reproductive success differed significantly ($P < 0.001$) from random expectations. Furthermore, males had significantly ($P < 0.001$) higher reproductive skew than females at both FT3/4 (males, $B = 0.049$, females, $B = 0.014$) and FT5 (males, $B = 0.089$; females, $B = 0.01$). Finally, the reproductive skew for males at site FT5 was significantly higher than the reproductive skew for males at FT3/4 ($P < 0.001$).

Specific examples

The genetic data revealed numerous additional details about who mated with whom to produce various progeny. For example, at site FT5 one male (FTb04) mated successfully with 13 of the 42 females (31%) present in that pool. Not only was he successful in terms of mate number, but his contributions to multiple-sire broods were usually highest (accounting for two-thirds or more of all offspring within each of nine broods). Overall, male FTb04 sired 242 of the 849 offspring (29%) born from the FT5 site. The next most successful male (FTb17, who mated with five females), sired another 118 offspring (14%) at the FT5 site. The remaining progeny at the FT5 site were split among 33 other sires. The high reproductive success of FTb04 and FTb17 cannot be explained merely by their continuous presence in the pool during the study period, because five other long-term resident males in this pool produced fewer progeny collectively ($n = 115$) than did FTb17 alone. Mating and reproductive skews were very pronounced in a subsample comprising long-term resident males ($P < 0.01$ and $P < 0.001$, respectively).

The situation was different at site FT3/4, where all five long-term resident males had more evenly distributed mating and reproductive successes. There, the number of mates per resident male ranged from four to seven, and the number of progeny ranged from 64 to 94. These males (captured in both 2006 and 2007) produced more progeny collectively than did the other six males that were caught in the FT5 pool only once (2006 or 2007). Neither mating nor reproductive skews in subsamples of long-term resident males were distinct from those expected under random occurrences. On the other hand, the number of mates and reproductive outputs were significantly skewed if all local males were considered ($P < 0.001$).

Discussion

Knowledge about genetic mating systems in nature is important for studies in population dynamics (such as assessing effective population size) and behavioural ecology (such as measuring the opportunity for sexual selection). For swordtails in particular, there is a special interest in understanding how sexual selection may have produced exaggerated secondary sexual traits (including long swords)

in males of this species. High variances in reproductive output, and correlations of reproductive success with mating success, are signatures of sexual selection (Arnold 1994). Our study is among the few available attempts to estimate male (as well as female) mating success and reproductive success jointly in a natural population of fish.

Reproductive skew in Xiphophorus helleri

We consistently found significant skews in reproductive success (and sometimes in mating success) in green swordtails, meaning that variances in progeny numbers were higher than expected from stochastic variation in fecundity. Furthermore, reproductive skew was significantly higher in males than in females, and a positive correlation existed between mate numbers and reproductive output in males but not females. A dependence of offspring production on mating success is a hallmark of sexual selection, and is consistent in this case with the inference that heritable traits under sexual selection tend to evolve more readily in male swordtails than in females. Our next step (C.I.M. Healey *et al*. in preparation) will be to search for correlations between reproductive success and various male phenotypes (sword length, body size, sword colour, etc.) that may allow us to specify particular features that are under sexual selection in our study population of this species (see also Rosenthal & Evans 1998; Trainor & Basolo 2006).

Reliability of our estimates of reproductive skew in males depends critically on whether we were able to collect nearly all of their progeny. Our sampling design, analyses, and interpretations are based on the assumption that the pools we sampled were effectively closed systems during this study. If not, the greater variance in male than in female mating success, as well as the stronger correlation between mating success and reproductive success in males than in females, could be a sampling artefact. Our genetic assessment of paternity showed that about 20–27% of all progeny were sired by males caught outside the focal pools. These males could have sired offspring during short visits to the focal pools, and might also have sired additional progeny elsewhere. Similarly, females captured in the focal pools could in some cases have mated outside. Indeed, the 2007 dry season was a particularly wet dry season and the pools in firetail Creek remained more connected than seen since we began our field research in the Bladen Branch River in 2001 (C.I.M. Healey, personal observation).

One way to limit possible bias introduced by outsiders is to consider only local males, that is, males captured at the focal sites. If we assume that such males did not mate outside of the pools, and that emigration by females was not extensive, we might then get a secure estimate of the variance in reproductive skew. Consideration of the local males is also convenient because it allows inclusion of the males who failed to leave any offspring. In analyses of local males only, we found significant reproductive skew in mating and reproductive success at both focal sites (positive skew index B, $P < 0.001$). In fact, skew in samples of local males was higher than in samples of all successful males (results not shown), presumably because the latter did not include males that failed to reproduce. Therefore, we think that our results present a conservative estimate of reproductive and mating skew in males. The correlation of mating and reproductive success was significant for successful local males (FT34, $r_S = 0.85$, $n = 11$, $P = 0.001$; FT5, $r_S = 0.53$, $n = 19$, $P = 0.02$).

Perhaps, the least biased estimate of variance in mating and reproductive success could be obtained by considering only long-term resident males, that is, those males who were captured in pools in both seasons. Most probably, these males stayed in the pools continuously for the same time and thus had equal opportunities to compete for females. The only possible bias would be from emigrating females or females that we failed to catch, but the bias should not be strong because such emigration should be random with respect to males. The drawback of the estimate is small sample size ($n = 5$ in FT3/4 and $n = 7$ in FT5). Analysis of these groups of males detected significant mating and reproductive skew in FT5, but not in FT3/4. The correlation of mating and reproductive success was not significant in either pool separately (possibly due to small sample sizes), but it was significant in the combined sample ($r_S = 0.74$, $n = 12$, $P = 0.006$).

Could the apparent extreme skew of mating and reproductive success by FTb04 (and some other males) be explained by the presence in the population of two or more males of identical or closely similar genotype? This seems unlikely for several reasons. First, the population gives no indication of being highly inbred, because genetic variation was clearly extensive and the loci were in accord with HWE. Second, given these facts, the probability that two randomly chosen individuals were identical by chance across all nine loci was extremely low (5×10^{-15}), and even random pairs of full siblings had a low average expected probability (3.6×10^{-5}) of being identical at these microsatellite loci. Third, although non-identical individuals can produce identical gametes on occasion, such an explanation can be refuted as a major source of parental assignment errors, as illustrated by the following example. In each of 11 broods at site FT5, 10 or more progeny were assigned to male FTb04. The rather large number of offspring in these broods allowed us to reconstruct the multilocus genotype of the sire (FTb04) and confirm that his alleles segregated in accord with Mendelian rules, thus making it improbable that the gametes in question could have come from other, non-identical specimens.

Despite the presence of a few big 'winners' (notably FTb04) in the reproductive sweepstakes at our study sites, most of the adult males reproduced. Altogether, only 11 of

the 41 local males with mature gonopodia (27%) failed to leave any progeny during the period of study. Among those 11 'losers', seven either had broken swords or swords that were not yet fully developed, suggesting that the males who failed to leave progeny were either hampered or had matured just recently. Thus, we envision not a mating system in which one or a few males usurp all available mates, but instead a system in which most males produce at least some progeny if they reach sexual maturity. This is facilitated by two features of the *X. helleri* mating system: (i) a pronounced bias (> 2:1) in the adult female : male sex ratio at our study sites (a female biased sex ratio in *X. helleri* has also been reported elsewhere; Franck *et al*. 1998); and (ii) common multiple mating by females.

In interpreting our current findings in the context of the opportunity for sexual selection on males, one caveat is that our results are based on the adult-to-fry reproductive success, yet the majority of fry clearly will not survive to reproductive age. In the future, this problem could be ameliorated by examining adult-to-adult reproductive success. Another major caveat is that we have estimated reproductive success during a single breeding season rather than across each individual's reproductive lifespan. Thus, in future it would be especially desirable to extend such studies across several years. This might be logistically feasible (albeit difficult), because males (and perhaps females also, after giving birth) could in principle be returned to their pools of origin after capture, with only small tissue samples removed for the appraisals of genetic parentage.

Comparisons with other settings

Luo *et al*. (2005) found multiple paternity in 28% of the broods in another swordtail species (*Xiphophorus multilineatus*), compared to multiple-paternity frequencies of 42–66% in related platyfish (also in the genus *Xiphophorus*) that do not have swords. The authors proposed that the low frequency of multiple paternity in swordtails vis-à-vis platyfish might be related to strong sexual selection due to the presence of swords. In our study, however, *X. helleri* had a significantly higher frequency of multiple paternity (64%) than *X. multilineatus* (Fisher's exact test, $P = 0.007$), thus not directly supporting Luo *et al*.'s proposition. However, perhaps the magnitude of sexual selection varies among *Xiphophorus* swordtail species or even among conspecific populations (as has been demonstrated in another poeciliid fish, *Heterandria formosa*; Soucy & Travis 2003). In any event, even if the proportion of females that mated with more than one male is exceptionally high in *X. helleri*, it remains lower than in reports for some other live-bearing fishes (*Gambusia holbrooki* and *Poecilia reticulata*) where 90–95% of broods were multiple sired (Zane *et al*. 1999; Hain & Neff 2007).

Luo *et al*. (2005) also demonstrated that paternity within each brood of *X. multilineatus* was skewed, with the most successful sires producing 67–80% of all offspring. In our current study of *X. helleri*, the within-brood skew in paternity was similarly high: on average, about 80% of the offspring came from the most successful of multiple sires. Paternity skew within broods can occur for variety of reasons, including precopulatory and postcopulatory mechanisms. Our observation that one male (FTb04) sired at least two-thirds of all offspring in each of 9 of 11 multisire broods suggests that sperm competition or sperm choice by females may be one of the mechanisms contributing to male reproductive skew.

With respect to the observed skew in paternity, where does *X. helleri* fall in the broader framework of comparable parentage analyses for other animal taxa? One way to standardize and quantify results among disparate organisms (including those with widely different fecundities) is via the standardized variance in breeding success (I_m), defined as the variance in male reproductive output divided by the square of the average male reproductive success (Wade & Arnold 1980). This metric can also be interpreted as a standardized measure of the opportunity for selection on males, and thus is a potential predictor of where a population might fall along a monogamy–polygyny continuum of mating systems, or along a gender monomorphism–dimorphism scale in sexually selected traits (Vanpé *et al*. 2008).

Table 4 displays the I_m value for *X. helleri* in a rank-ordered list of I_m values previously reported from comparable parentage analyses in several other animal taxa. From this perspective, the opportunity for sexual selection on male *X. helleri* is intermediate between highly polygynous and sexually dimorphic species such as bighorn sheep and elephant seal, and relatively monogamous and monomorphic species such as roe deer.

The relatively high I_m value of 2.5 suggests that there is strong potential for natural and/or sexual selection on males in *X. helleri*. In theory, the standardized variance in reproductive success places an upper bound on the change in mean fitness of a population in the next generation (Shuster & Wade 2003), although the realized change ultimately will depend on fitness heritability as well. With $I_m = 2.5$, the mean population fitness should increase if the heritability of any phenotypic trait underlying fitness is above 40%.

Available estimates of I_m in fish mostly come from experimental or seminatural arrangements (Fleming & Gross 1994; Becher & Magurran 2004; Spence *et al*. 2006; Reichard *et al*. 2007); they range from 0.07 in zebrafish to 2.1 in bitterling and coho salmon. The highest estimates of I_m have been obtained in conditions when competition for females was arranged to be high (Fleming & Gross 1994; Reichard *et al*. 2007). Only a few studies have evaluated individual

Table 4 Examples of I_m values (standardized variances in male breeding success) in populations of several animal species that have been intensively scrutinized for paternity using genetic markers or field observations. The reported I_m values are listed in rank order from largest (top of the list) to smallest (bottom). Unless specified otherwise, I_m values are based on reproductive success of sexually mature males. Mean I_m values are given for each study where estimates are available for more than one population

Species	Common name	I_m	Reference
Ovis canadensis	Bighorn sheep	4.52	Coltman *et al.* 2002
Mirounga angustirostris	Elephant seal	3.91	Le Boeuf & Reiter 1988
Rhinolophus ferrumequinum	Horseshoe bat	3.70	Rossiter *et al.* 2006
Ovis aries	Soay sheep	3.46	Coltman *et al.* 1999
Xiphophorus helleri	Green swordtail	2.50	Current study
Antechinus stuartii	Brown antechinus (a marsupial)	2.01	Holleley *et al.* 2006
Antilocarpa americana	American pronghorn	1.87	Byers 1997
Antechinus agilis	Agile antechinus (a marsupial)	1.82	Kraaijeveld-Smit *et al.* 2003
Cervus elaphus	Red deer	1.43	Clutton-Brock *et al.* 1988
Capreolus capreolus	Roe deer	0.75	Vanpé *et al.* 2008
Salmo salar	Atlantic salmon	0.59	Garant *et al.* 2001
Leptonychotes weddellii	Weddell seal	0.33*	Harcourt *et al.* 2007
Eubalaena glacialis	Right whales	0.22*	Frasier *et al.* 2007
Megaptera novaeangliae	Humpback whale	0.20*	Cerchio *et al.* 2005
Phoca vitulina	Harbour seal	0.16*	Hayes *et al.* 2006

*Estimate based on successful males only.

reproductive success in natural populations (and not all of them report the variances and means necessary to calculate I_m). In one such study, Garant *et al*. (2001) reported a value of 0.59 for Atlantic salmon. Thus, available estimates of the standardized variance in reproductive success suggest that the opportunity for selection in *X. helleri* may be among the highest yet reported in fishes.

The conspicuous sword in *X. helleri* males is considered to be a result of sexual selection due to female choice. We found high skews in reproductive success in both natural pools examined, but it remains to be determined whether features of the sword or any other phenotypic characteristics were implicated in producing such skews. Establishing whether there is association of particular phenotypic features of *X. helleri* with reproductive success is a next obvious research step.

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