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Journal

Evolution, 51(5)

ISSN

0014-3820

Authors

Jones, Adam G
Avisé, John C

Publication Date

1997-10-01

DOI

10.1111/j.1558-5646.1997.tb01484.x

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POLYGYNANDRY IN THE DUSKY PIPEFISH *SYNGNATHUS FLORIDAE* REVEALED BY MICROSATELLITE DNA MARKERS

ADAM G. JONES¹ AND JOHN C. AVISE

Department of Genetics, University of Georgia, Athens, Georgia 30602

Abstract.—In the dusky pipefish *Syngnathus floridae*, like other species in the family Syngnathidae, ‘pregnant’ males provide all post-zygotic care. Male pregnancy has interesting implications for sexual selection theory and the evolution of mating systems. Here, we employ microsatellite markers to describe the genetic mating system of *S. floridae*, compare the outcome with a previous report of genetic polyandry for the Gulf pipefish *S. scovelli*, and consider possible associations between the mating system and degree of sexual dimorphism in these species. Twenty-two pregnant male dusky pipefish from one locale in the northern Gulf of Mexico were analyzed genetically, together with subsamples of 42 embryos from each male’s brood pouch. Adult females also were assayed. The genotypes observed in these samples document that cuckoldry by males did not occur; males often receive eggs from multiple females during the course of a pregnancy (six males had one mate each, 13 had two mates, and three had three mates); embryos from different females are segregated spatially within a male’s brood pouch; and a female’s clutch of eggs often is divided among more than one male. Thus, the genetic mating system of the dusky pipefish is best described as polygynandrous. The genetic results for *S. floridae* and *S. scovelli* are consistent with a simple model of sexual selection which predicts that for sex role-reversed organisms, species with greater degrees of sexual dimorphism are more highly polyandrous.

Key words.—Cuckoldry, mating success, parentage analysis, polyandry, sex role reversal, sexual dimorphism, sexual selection.

Received January 2, 1997. Accepted May 7, 1997.

Male pregnancy, otherwise rare in the biological world, is ubiquitous in fishes of the family Syngnathidae and has interesting evolutionary implications. Pregnant males necessarily provide extensive parental care. Thus, either as a direct result of parental investment strategies (Trivers 1972), or by a reduction in the reproductive rate of males (which produces a female-biased operational sex ratio; Emlen and Oring 1977; Clutton-Brock and Parker 1992), females may find themselves in the unusual position of having to compete for access to mates. Conversely, males may have a luxury of being choosy (Rosenqvist 1990; Berglund and Rosenqvist 1993). This sex-role reversal provides opportunities to test theories of sexual selection from a unique perspective (Williams 1975).

The family Syngnathidae is comprised of more than 200 species of pipefishes and seahorses. During copulation, a female transfers eggs to a male’s external ventral surface or internal brood pouch (depending on the species), where he fertilizes them and provides the developing embryos with nutrients, osmoregulation, aeration, and protection until they are live-born (Haresign and Shumway 1981; Berglund et al. 1986). Male investment varies among syngnathid species, as do apparent mating behaviors and degrees of sexual dimorphism (Vincent et al. 1992). Thus, Syngnathidae is an excellent group to investigate possible associations between the evolution of secondary sexual characters and the mating system in role-reversed taxa.

Darwin (1871) originally suggested that highly polygynous species with “traditional” sex roles tend to be more sexually dimorphic than less polygynous species. Previous field studies of such associations in birds and mammals have yielded mixed results (e.g., Clutton-Brock et al. 1977, 1980; Payne 1984; Höglund 1989; Björklund 1990; Oakes 1992). One potential problem with field studies, however, is that they

rely upon observed copulations that may inadequately assess the genetic mating system upon which sexual selection theory is based (Harvey and Bradbury 1991; Pemberton et al. 1992; Avise 1996). Field studies often ignore extra-pair paternity and use the social mating system as a correlate to sexual dimorphism (Webster 1992; Winquist and Lemon 1994). Yet, recent genetic analyses have demonstrated that extra-pair paternity (reflecting genetic polygyny or polygynandry) is common in many avian species formerly thought to be monogamous (Lack 1968) by behavioral criteria (reviewed in Birkhead and Møller 1992; Avise 1994; Westneat and Webster 1994). Discrepancies between the socially estimated and the genetically defined mating systems may help to explain the difficulties in correlating mating systems with sexual selection intensity (e.g., Møller and Birkhead 1994; Webster et al. 1995).

An analog of Darwin’s aforementioned theory is that in species with reversed sex roles, more polyandrous species are expected to be more dimorphic in secondary sexual traits than species that exhibit a less polyandrous mating system. However, relatively little theoretical or empirical attention has been directed toward this predicted relationship (Jehl and Murray 1986; Andersson 1995), probably because sex role reversal is rare. The study of sex role-reversed organisms promises to enhance our understanding of sexual selection as an evolutionary process (Williams 1975), but highly polymorphic molecular markers seldom have been applied to investigate the genetic mating systems of species with reversed sex roles (but see Oring et al. 1992). An important step in addressing the predicted relationship between polyandry and sexual dimorphism would be to describe the genetic mating systems of additional sex role-reversed taxa. The discovery of microsatellite markers (Tautz and Renz 1984; Tautz 1989) has opened new possibilities for the genetic assessment of parentage and mating systems in nature (e.g., Brockmann et al. 1994; Craighead et al. 1995; Kellogg et al. 1995; Primmer et al. 1995; Colbourne et al. 1996).

¹ Corresponding author.

A. *micro25.10* maternal alleles

	150	160	146	182
96	3	7	0	0
100	11	7	0	0
102	0	0	1	2
108	0	0	7	4

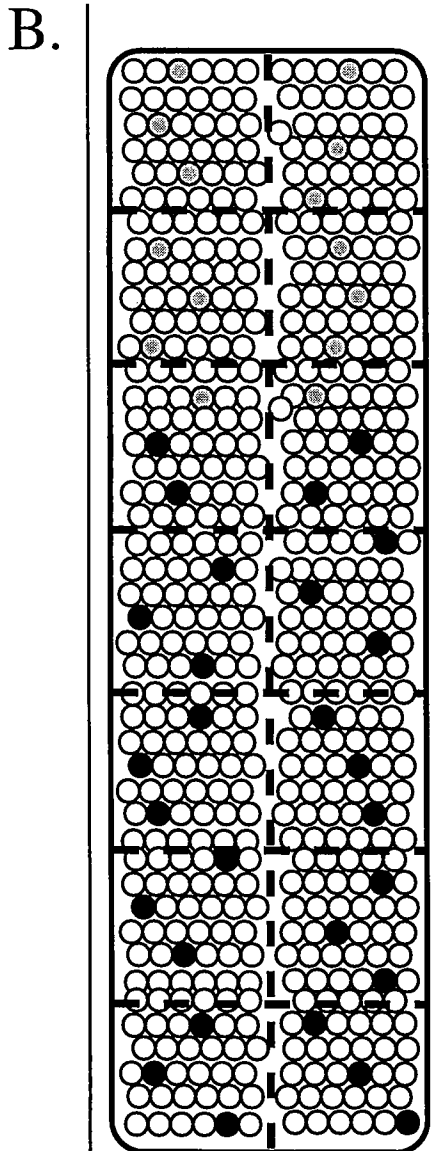


FIG. 1. Example of the procedure for assignment of maternal two-locus genotypes to embryos in a male's brood pouch. (A) First, the allelic contribution of the father (in this case, DM9-13) is subtracted to determine each embryo's maternally-derived alleles. A table of

Here we employ microsatellite markers to assess the genetic mating system of the dusky pipefish *Syngnathus floridae*, which lacks obvious secondary sex characters. Our approach has been to compare genotypes of pregnant males with those of their offspring and thereby infer the maternal genotype(s) of those embryos. The proximate aims are to identify the numbers of females who have contributed eggs to each male's brood; assess the spatial position within brood pouches of multiply mothered clutches of embryos; assess the possibility of cuckoldry by males (as a test of a theoretical prediction that the evolution of high investment in offspring by males is facilitated by an assurance of genetic paternity; Smith 1979); reveal how often individual females distribute batches of eggs to more than one male; and compare all outcomes with those previously reported by Jones and Avise (1997) for a sympatric congener that is more dimorphic in secondary sexual characters, the Gulf pipefish *S. scovelli* (Brown 1972). The broader intent is to contribute to a developing data base on genetic parentage in syngnathid fishes with a view toward addressing possible relationships between the mating system, sexual selection, and sexual dimorphism in sex role-reversed species.

MATERIALS AND METHODS

The four microsatellite loci employed in this study originally were cloned from the Gulf pipefish as described by Jones and Avise (1997). Tissue preparation and PCR conditions were modified slightly from the original study, as follows. The reactions were carried out in 10 μ l volumes containing 1x Promega *Taq* buffer, 1.5 mM MgCl₂, 0.15 μ M of each primer, 0.1 mM of each dNTP, and 0.5 units Promega *Taq* polymerase. One PCR primer was end-labeled with 1 μ Ci γ ³²P ATP per 5 pmol of primer. The cycling parameters were as reported in Jones and Avise (1997) and microsatellite products were resolved on standard 6% polyacrylamide denaturing sequence gels. Two of the microsatellite loci (*micro25.10* and *micro25.22*) were amplified simultaneously by adding the second set of primers to the standard PCR mixture. Alleles were scored and designated according to the numbers of base pairs (bp) in the amplified fragment.

Fifty adult males and 43 adult females of the dusky pipefish were collected by seine on 1, 7, and 8 July 1994, in shallow (< 1.5 m) seagrass meadows along approximately 100 m of shoreline of the St. Joseph State Park (Gulf Co.) on the Gulf Coast of Florida. Fish were frozen on dry ice and returned to the laboratory. Of the adult males, 43 were pregnant and 22 carried embryos sufficiently developed for microsatellite

←
embryo counts then is constructed with maternally inferred alleles from the two respective loci placed along the top and side of the table. In this case, the alleles 150 and 160 at the *micro25.10* locus are associated with alleles 96 and 100 at *micro25.22*, whereas the *micro25.10* alleles 146 and 182 are associated with the *micro25.22* alleles 102 and 108. Thus, the two-locus genotype of each mother becomes apparent. (B) Schematic representation of the brood pouch of pregnant male DM9-13 showing the sampling design and spatial partitioning of the sets of embryos (black vs. gray) from the two inferred mothers. Eggs actually were stacked but for simplicity are depicted here as nonoverlapping.

TABLE 1. Microsatellite locus designations and sample sizes (*n*) of presumably unrelated adults used to establish baseline genetic information from the St. Joseph State Park collection of dusky pipefish. *P*-values are given for exact tests for departures from Hardy-Weinberg expectations. Per-locus exclusion probabilities are the expected fractions of females excluded from maternity given a single father-offspring pair. For comparison, the same information is included for the population of Gulf pipefish from which the markers originally were cloned (Jones and Avise 1997).

Locus	<i>n</i>	No. of alleles	Heterozygosity		Exact test <i>P</i>	Exclusion probability
			Observed	Expected		
micro11.1						
Gulf pipefish	42	22	0.929	0.922	0.340	0.844
Dusky pipefish	56	44	0.910	0.942	0.166	0.917
micro22.3						
Gulf pipefish	42	19	0.952	0.916	0.280	0.830
Dusky pipefish	56	25	0.946	0.916	0.793	0.864
micro25.10						
Gulf pipefish	78	28	0.885	0.892	0.226	0.796
Dusky pipefish	65	18	0.785	0.852	0.054	0.740
micro25.22						
Gulf pipefish	81	29	0.877	0.947	0.011	0.892
Dusky pipefish	65	21	0.877	0.902	0.045	0.830

assay. The sampling scheme for embryos was as follows. First, each brood pouch was divided into 14 sections and marked (Fig. 1B). The outer membrane of the brood pouch was dissected away, and from each section three embryos (for a total of 42 embryos per male) were plucked by forceps and their positions recorded. Each embryo was rinsed in deionized water and placed individually in an eppendorf tube for PCR template preparation (described in Jones and Avise 1997) and microsatellite analysis. All embryos and adults were assayed for micro25.10 and micro25.22. The other two loci (micro11.1 and micro22.3) were used only in cases of ambiguity and to establish four-locus genotypic 'fingerprints' for females of special interest (see below).

RESULTS

Microsatellite Loci

The four PCR primer pairs originally developed for the Gulf pipefish also revealed polymorphic loci in the dusky pipefish. At micro25.10 and micro25.22, fewer alleles were observed in the dusky pipefish than in the Gulf pipefish, whereas the reverse was true for the other two loci (Table 1). Most notable was micro11.1 where 44 alleles were observed in the dusky pipefish but only 22 were present in the Gulf pipefish sample from the same locale. Heterozygosities and parental exclusion probabilities are comparable in the two species for the four loci under consideration. The allelic frequency distributions differed between the species (Fig. 2), but variability in both collections was extremely high. Thus, these loci are excellent for mating system and parentage analysis: combined four-locus expected exclusion probabilities (Chakraborty et al. 1988) are greater than 0.999 for both species.

In the dusky pipefish, a null allele at low frequency was

observed at micro22.3: This allele manifested itself clearly in a pregnant male (DM7-2) who scored as homozygous for the allele 171 but by appearances did not pass the allele to several of his progeny. However, this null allele was infrequent enough in the population so as not to cause a heterozygosity deficit at this locus (Table 1). Another conceivable explanation for the inheritance pattern of this null allele would be that it represents a case of cuckoldry. In this case, however, cuckoldry is not a likely explanation, because DM7-2 could not be excluded as a father of the embryo based on any of the other three loci assayed. Thus, if he was cuckolded it was by a male that shared his exact genotype at three of the loci (expected frequency of 1.3×10^{-7}) and also shared one of his alleles at the fourth locus. This danger of null alleles at microsatellite loci should caution against exclusion in cases in which both the father and offspring are scored as homozygous.

The exact test of Guo and Thompson (1992) as implemented in the program GENEPOP (Raymond and Rousset 1995) was used to test for departures from Hardy-Weinberg (H-W) equilibrium in the collections of adults (Table 1). Of the four loci, only micro25.22 deviated significantly from H-W expectations. In the Gulf pipefish this deviation can be explained by the presence of null alleles (Jones and Avise 1997), although nulls were not observed at this locus in the dusky pipefish. In any event, the microsatellite markers did not deviate from expected Mendelian genotypic counts in progeny arrays within broods ($\alpha = 0.05$, χ^2 corrected for multiple comparisons).

Mating System of Males

From the observed genotypes, in no case could a pregnant male be excluded as a potential father of any of the 42 assayed embryos in his brood, strongly suggesting that no fertilizations by foreign sperm (male cuckoldry) had occurred in our sample of 22 males. Thus, the two-locus genotype of each father compared with those observed in his brooded progeny allowed the maternal allelic contribution to each embryo to be determined by subtraction (Table 2). The logic used to deduce the exact two-locus genotype of the mother of each embryo within a brood is exemplified in Figure 1A. Among the progeny in the brood pouch of pregnant male DM9-13, four maternal alleles (146, 150, 160, and 182) were observed for locus micro25.10 and another four alleles (96, 100, 102, and 108) were observed for locus micro25.22. The collective presence of four maternally derived alleles at either locus immediately indicates that this male (DM9-13) had mated with at least two females. The gametic phase disequilibrium evident in the body of the matrix in Figure 1A makes the two-locus genotype of the mother of each embryo apparent. Thus, the maternally derived alleles 150 and 160 at micro25.10 always were associated with alleles 96 and 100 at micro25.22, strongly suggesting that a single female with the two-locus genotype 150/160 (micro25.10), 96/100 (micro25.22) had mothered these embryos. From the same reasoning, DM9-13's other mate had the genotype 146/182, 102/108. Other explanations for these genotypic distributions are conceivable, but given the rarity of homozygotes and of in-

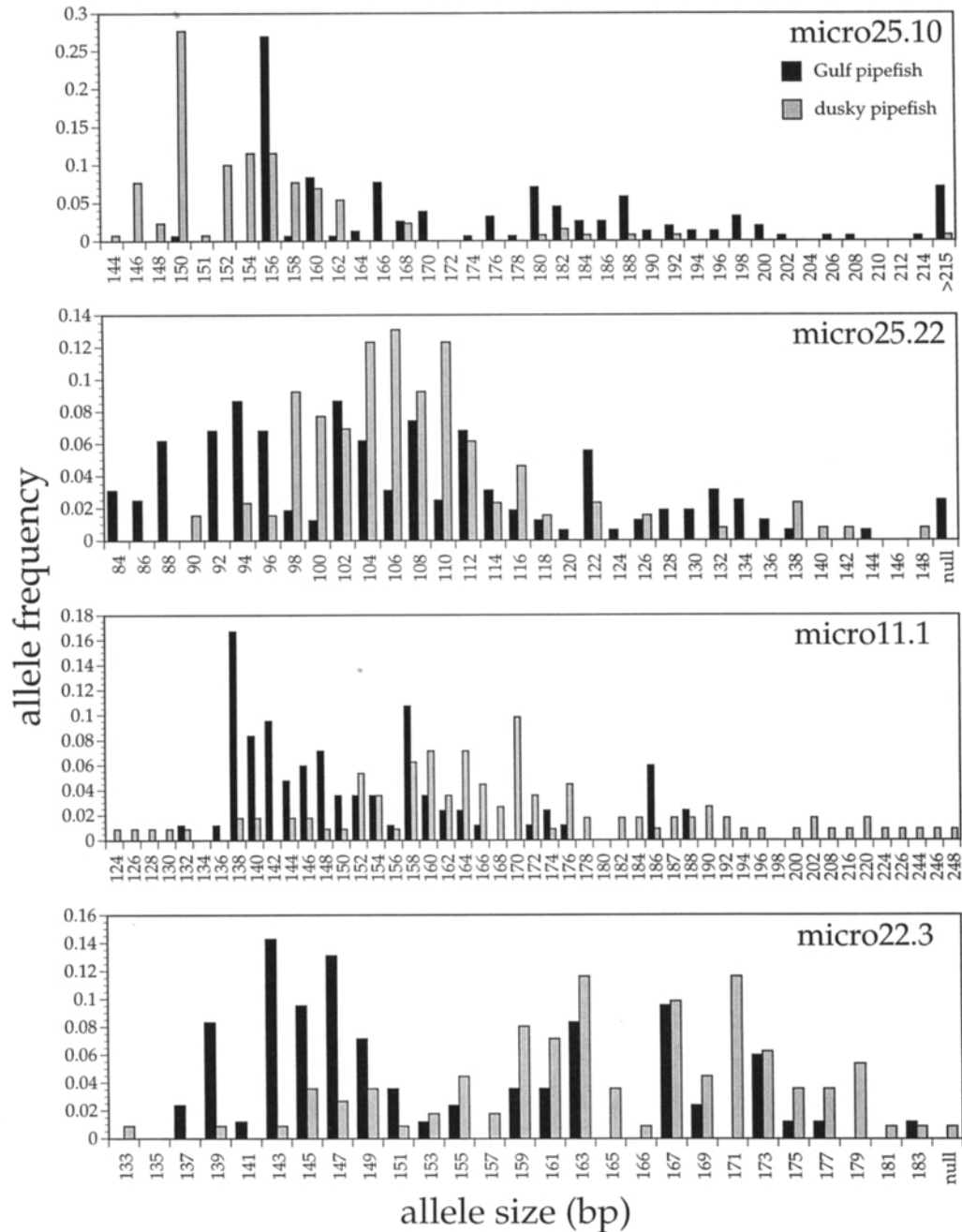


FIG. 2. Histograms of population allele frequencies at each of four microsatellite loci in the northern Florida collection of dusky pipefish (gray bars) and Gulf pipefish (solid bars). These samples were derived from presumably unrelated adults collected for both species simultaneously from the same seagrass meadows.

dividuals that share the same genotype by chance, these are unlikely to be correct.

The embryos from each mother then were mapped within each brood pouch (Figs. 1B, 3). In the case of DM9-13, the posterior-most 28 embryos scored were mothered by one female and the 14 anterior-most embryos by another. Thus, embryos were spatially clumped by maternity within the brood pouch such that full siblings were grouped together. This spatial grouping of embryos held true for all 16 multiply mated males assayed in this study (Fig. 3). Within a male, embryos invariably were at a similar developmental stage,

indicating that the egg transfers probably took place within a short time span relative to the length of male pregnancy.

Among the 22 males, six had mated with a single female, 13 had two mates, and three had three mates (Table 2; Fig. 3). There was a tendency for larger males to have a larger number of mates (Fig. 4). There also was a significant positive association between a male's length and the number of embryos in his brood pouch (Fig. 5), which is not surprising given that larger males have larger brood pouches. The overall distribution of male mating success is given in Figure 6A.

The fact that embryos are clumped spatially within the

TABLE 2. Genetic descriptions of 22 pregnant male dusky pipefish, and of the inferred mothers for their respective broods of embryos. Females are shown by mating order with each first row describing a male's initial mate (most posterior egg mass) and the last row his final mate (most anterior egg mass). The final column lists the adult females implicated as mates that were present within our collected sample. The four females in bold each contributed eggs to two males in our sample (as verified by data from an additional pair of microsatellite loci—see text). Mothers with the designation 'nc' were not collected in our sample of 65 adults.

Father's I.D.	Father's genotype				Inferred mother's genotype				# eggs given	Mother's I.D.
	<i>micro25.10</i>		<i>micro25.22</i>		<i>micro25.10</i>		<i>micro25.22</i>			
Males with one mate										
DM8-9	146	148	112	122	150	152	108	108	369	nc-1
DM9-11	150	150	112	114	158	182	98	104	415	nc-2
DM9-7	146	146	108	110	150	156	104	126	—	nc-3
DM9-4	150	152	98	102	148	150	102	110	200	nc-4
DM8-10	154	154	100	126	158	160	104	114	—	nc-5
DM10-4	156	158	138	138	160	160	106	122	342	DF9-1
Males with two mates										
DM10-5	152	156	90	100	160	160	106	122	256	DF9-1
					150	151	110	122	342	DF9-8
DM9-10	146	160	106	112	154	156	106	106	288	nc-6
					150	150	98	98	214	DF10-3
DM9-3	150	160	100	106	154	156	100	104	490	nc-7
					154	156	100	106	204	DF10-2
DM9-9	150	152	112	116	146	150	94	112	563	nc-8
					150	152	98	104	75	nc-9
DM9-6	150	154	98	116	150	160	96	100	402	DF10-9
					150	150	98	108	390	nc-10
DM9-13	146	160	106	110	150	160	96	100	365	DF10-9
					146	182	102	108	159	nc-11
DM10-2	150	158	104	116	138	158	98	118	260	nc-12
					146	154	110	110	189	DF10-5
DM10-10	150	150	94	116	152	158	98	136	73	nc-13
					146	154	98	106	148	nc-14
DM10-6	150	152	98	148	146	150	98	106	174	nc-15
					150	158	108	120	122	nc-16
DM7-3	156	184	108	108	152	162	100	100	353	DF7-3
					150	164	100	108	252	nc-17
DM7-5	150	152	104	110	148	150	104	108	165	nc-18
					142	146	112	136	104	nc-19
DM8-1	148	154	98	102	160	190	108	112	380	nc-20
					154	156	110	132	252	
DM8-7	154	168	104	108	150	156	108	138	232	DF8-1
					150	150	94	98	337	nc-21
Males with three mates										
DM8-4	150	188	98	118	150	156	108	138	284	DF8-1
					160	190	108	112	197	nc-20
					150	150	100	114	125	DF8-4
DM7-1	156	158	104	104	154	160	98	104	331	nc-21
					152	154	96	106	406	nc-22
					150	150	94	110	155	DF7-7
DM7-2	156	156	102	110	156	218	104	110	190	DF8-3
					146	148	104	104	203	nc-23
					150	174	92	106	95	nc-24

brood pouch by mother allowed us to estimate the numerical contribution of embryos from each female (Table 2). This was accomplished by counting all embryos in each of the 14 demarcated sections of the brood pouch, and ascribing them to the particular mothers who produced the embryos that had been scored genetically from that section. Some error may be incurred at the transition zone from one mother to the next, since within sections of mixed maternity each female was assigned embryos by multiplying her proportion of embryos assayed in the section (i.e., 1/3 or 2/3) by the total

number of embryos in the section. Females produced a mean of 259 embryos per successful mating effort. This may be an underestimate of the number of eggs actually transferred because during a brood's development some eggs may die or be absorbed by males (Ahnesjö 1992, 1996).

Mating Behavior of Females

The high variability of the markers also permitted inferences about female mating behavior. Eleven adult females in

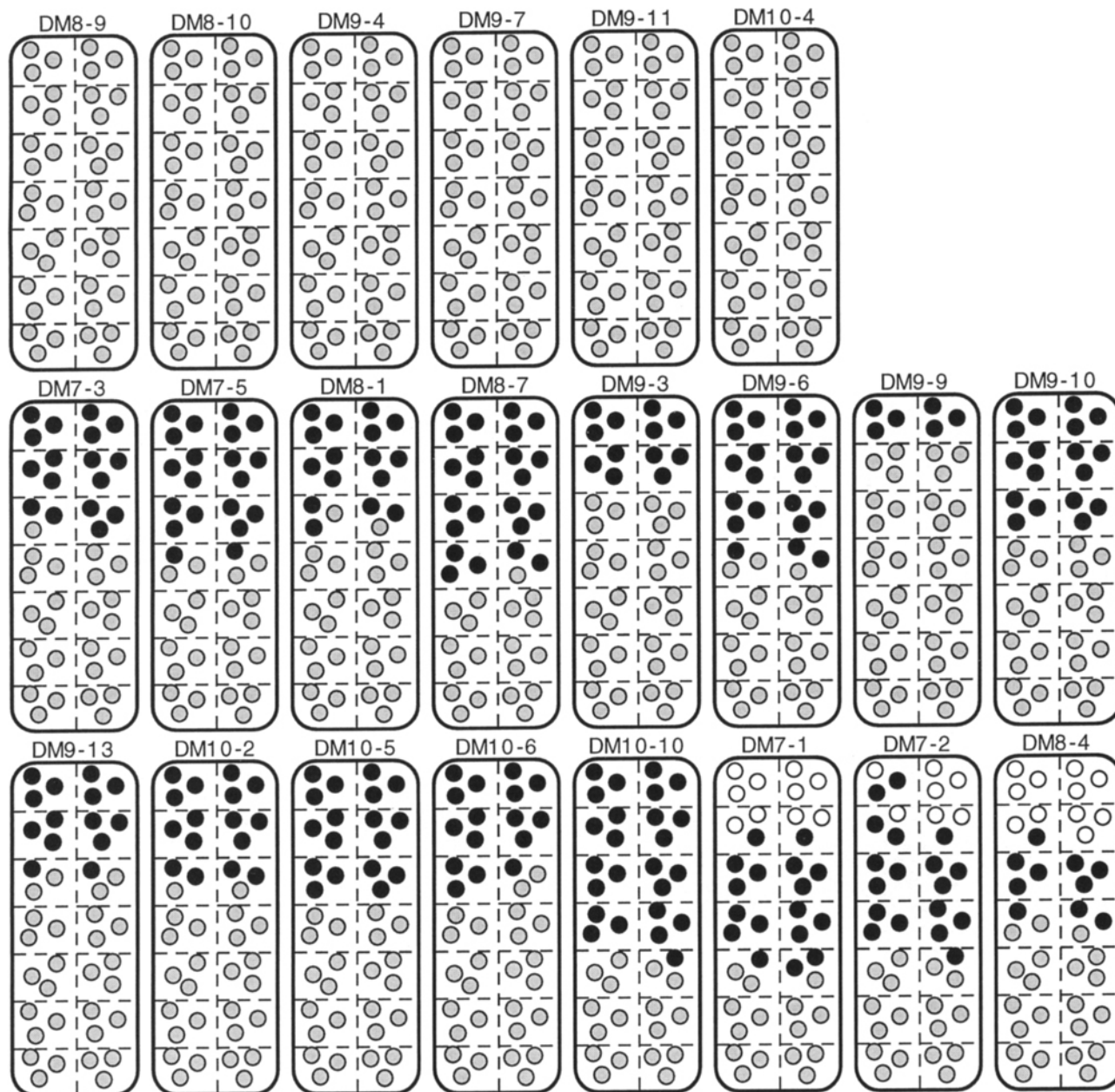


FIG. 3. Diagrammatic representation of the brood pouches of the 22 pregnant males assayed for the dusky pipefish. Each circle within a brood pouch represents an assayed embryo. Genotypes were inferred as in Figure 1. Based on these genetic descriptions, like-colored circles within each brood pouch represent full sibs; unlike-colored circles are half-sibs. Brood pouches are not drawn to scale; for estimated numbers of embryos contributed by each mother, see Table 2.

the collection shared identical two-locus genotypes with particular inferred mates of sampled males. These situations were investigated further by screening two additional loci (micro11.1 and micro22.3). Each case yielded a perfect four-locus match. Expected population frequencies of these multi-locus genotypes ranged from 10^{-8} to 10^{-10} (product rule based on H-W equilibrium), strongly implicating these particular females in our collection as mates of the assayed pregnant males. There was no significant correlation between female size and number of eggs transferred (regression, $P = 0.11$). However, these 11 females were longer on average;

(185.8 ± 9.5 mm [SD]) than the 32 other females (167.7 ± 16.8 mm) in the collection ($P < 0.001$, t -test).

We also detected multiple mating by females within our sample. On four occasions, inferred two-locus genotypes of mothers were shared by broods of two males (Table 2). For example, the only mate of pregnant male DM10-4 displayed a genotype identical to that of one of the two mates of DM10-5 (Table 2). In each such case, nine embryos per brood then were assayed at the two additional microsatellite loci; a perfect four-locus match for the inferred mother's genotype invariably resulted. Expected population frequencies of these

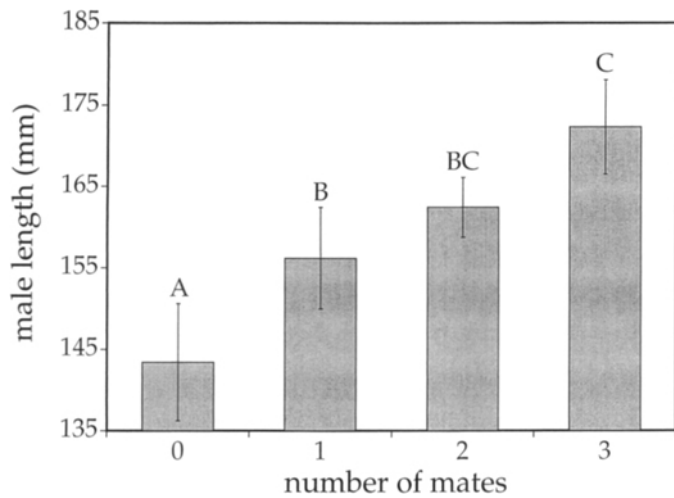


FIG. 4. Mean sizes of dusky pipefish males with zero mates ($n = 7$), one mate ($n = 5$), two mates ($n = 13$) and three mates ($n = 3$). Error bars represent 95% confidence intervals. Bars with like letters have means that were not significantly different (ANOVA, Fisher's PLSD, $\alpha = 0.05$).

multilocus genotypes ranged from 10^{-8} to 10^{-10} . In this manner, instances of multiple mating by females were deduced.

Three of these multiple-mating females (DF9-1, DF10-9, and DF8-1) were represented in our collection of 43 adult females (Table 2). Judging from the developmental stage of embryos within the different males, it appears that in some cases a female had split a single clutch of eggs among the two males within a short time span, whereas in other cases a female may have transferred batches of eggs at different times. For example, DM9-6 and DM9-13 both carried well-developed embryos with small yolks, so DF10-9 probably mated with these two males in a short time interval. In contrast, DM10-4 carried well-developed embryos with small yolks whereas DM10-5 carried early embryos (eyes barely

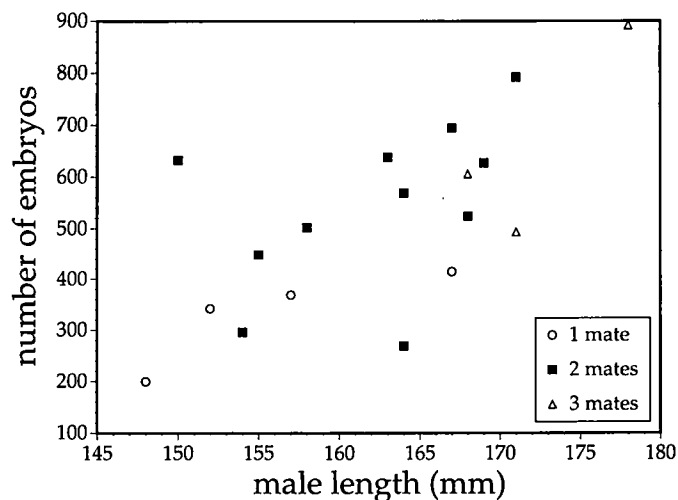


FIG. 5. Sizes of dusky pipefish males and numbers of embryos within the brood pouch. The relationship is significant ($P = 0.002$). Males that received eggs from one, two, and three mates are shown as open circles, closed squares, and open triangles, respectively.

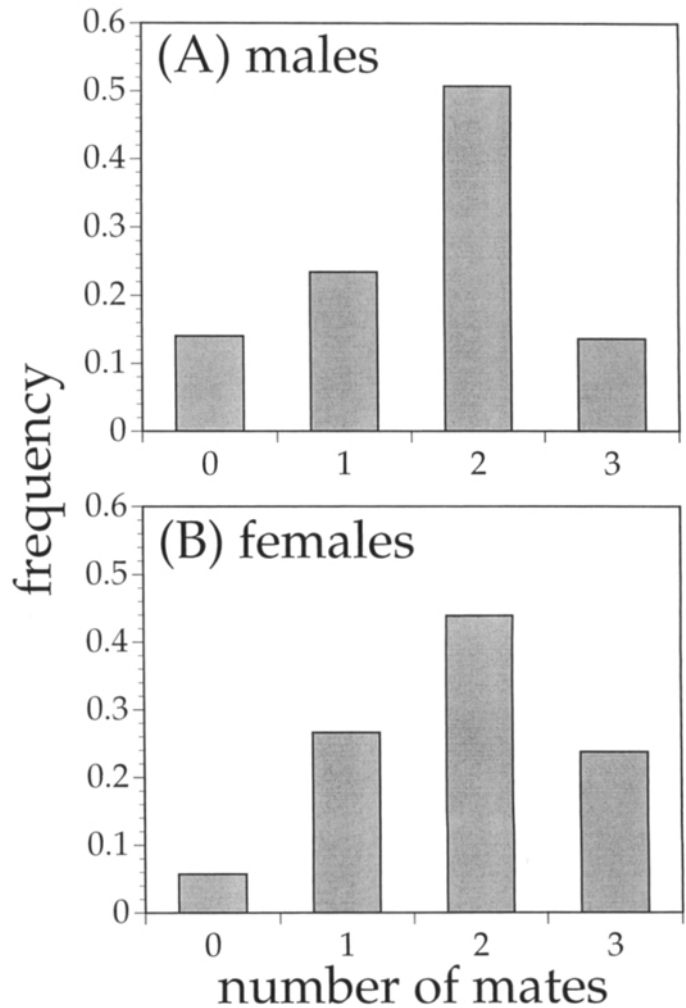


FIG. 6. Frequency histograms of mating success for males (A) and females (B). The histogram for males is derived from direct observations of the 22 pregnant males whose broods were analyzed, coupled with the consideration that 7 of the 50 males collected were not pregnant. The female mating success histogram is the best-fitting binomial distribution (mean = 1.86, var = 0.71), based on a model of random mating by females in the computer simulations (see text).

visible) with large yolks, indicating that DF9-1 first mated with DM10-4 and then at a later time with DM10-5.

DISCUSSION

The Genetic Markers

These microsatellite loci have proved extremely useful in parentage analyses in both *S. floridae* and *S. scovelli*. Other studies have reported an apparent reduction in the variability of microsatellites when the loci are assayed in species other than that from which they originally were cloned (Primmer et al. 1996). The similar performance of these loci in the dusky and Gulf pipefish might possibly reflect a relatively recent common ancestry for these species.

Null alleles at microsatellite loci are a general complication for parentage assessments in nature (Callen et al. 1993; Koorvey et al. 1993; Phillips et al. 1993; Pemberton et al. 1995),

but they did not pose a serious problem in the current study. Large numbers of progeny per parent were assayed, so any null alleles would have been obvious from non-Mendelian genotypic counts in progeny arrays. Only one locus (micro22.3) among those assayed in the dusky pipefish carried a null allele, and then only in a single heterozygous parent.

Cuckoldry

In no case in our sample of dusky pipefish could a brooding male be genetically excluded as the father of the offspring he carried. The high variability of the markers employed makes it extremely unlikely that any true instances of cuckoldry remained undetected, so the phenomenon must be rare in this species if it occurs at all. This is hardly surprising given the reproductive adaptations of *Syngnathus*. Although copulation has not been directly observed for *S. floridae*, all members of the genus have a similar pouch configuration, so visual observations of the transfer of eggs by *S. typhle* may be germane. During copulation in the latter species, eggs are deposited directly from a female's reproductive tract into a male by an ovipositor inserted through a small opening at the anterior end of his brood pouch (Ahnesjö 1992; Berglund and Rosenqvist 1993). The male fertilizes the eggs by sperm released inside the pouch and, thus, the eggs presumably are not exposed to potential fertilizations by foreign sperm.

Not all members of the family have enclosed pouches, however, so cuckoldry by 'sneaker' males remains a likelier possibility for some syngnathid species. For example, in the pipefish *Nerophis ophidion*, eggs are attached directly to the male's external ventral surface during copulation (Rosenqvist 1993). The male then releases sperm and fertilizes the eggs by floating through the sperm cloud. Other syngnathid males have brooding designs that range from external gluing of eggs, to partially open pouches, to complete pouch closure (Herald 1959).

Male Mating Behavior

In our sample, frequent multiple mating by dusky pipefish males was deduced from the observed genotypic arrays. It is worth considering two ways in which the number of mates per male might be underestimated by this approach. First, because of the large number of embryos per brood (mean $n = 505$), only a subset of offspring was screened genetically. However, in every detected case involving a multiply mothered progeny array, full sibling embryos proved to be clumped spatially in the brood pouch. This spatial arrangement makes it unlikely that we would fail to detect a contribution of as few as 10–15 eggs by an additional female (see Fig. 1B). Second, a male might have received eggs from two females with identical two-locus genotypes. This too is unlikely. Based on the product rule, the *most common* two-locus genotype for the sampled loci had an expected frequency of 2.5×10^{-3} , and no individuals in our collection of 65 adults shared a composite genotype.

In natural populations of *S. typhle*, multiple mating by males sometimes is registered when eggs of different colors co-occur within a male's brood pouch (Berglund et al. 1988). Such assays based on visual inspection would severely underestimate the frequency of multiple mating for *S. floridae*,

because in our sample of 22 pregnant males only once did morphological evidence suggest multiple mating. In male DM9-10, embryos in the posterior portion of the brood pouch had paler yolks than those in the anterior end. Genetic analysis confirmed that this color difference reflected an instance of multiple mating (Table 2).

Some of the variance in male mating success in this population could reflect the operation of sexual selection. Larger dusky pipefish males appear to have more mating partners than smaller specimens (Fig. 4), a finding consistent with an observation in *S. typhle* that females prefer to mate with larger males (Berglund and Rosenqvist 1993). However, a remaining question is what fraction of the variance in male reproductive success can be attributed to variance in mating success per se (Arnold 1983). Is male reproductive success limited by access to mates, or more so by intrinsic male brooding capacity or other factors?

The brood pouches of all pregnant males assayed appeared to be completely full of embryos. The only exception to this pattern was the male DM10-10, which appeared to have been giving birth at the time that he was collected. 'Partial fullness' should be obvious, because (at least in *S. typhle*) a male shakes down the eggs received from an initial mating to the posterior end of the brood pouch before mating with another female. The lack of partially filled brood pouches in *S. floridae* suggests that the reproductive rate of pregnant males in the assayed population is limited not by the mating success of males but rather by brooding capacity (a result consistent with historical thought on syngnathid taxa; Williams 1975). Thus, perhaps little of the variation in reproductive success of dusky pipefish pregnant males can be attributed to mate acquisition, in which case sexual selection on these males is expected to be weak (Andersson 1994).

Males who fail to mate could provide another potential source of variance in reproductive success (Arnold and Duvall 1994). We observed seven unmated individuals in our sample of 50 adult males. Several explanations are possible. First, some of these males may have been between broods, given that a time interval probably exists after parturition during which a male is not receptive (Vincent and Sadler 1995). Second, some of the smaller males may have appeared to be adults by the presence of a brood pouch, but were too young to mate or receive eggs. Third, perhaps unmated mature males were small, unattractive, or otherwise unable to procure mates. Among the three possibilities, only the latter could be taken as strong evidence for sexual selection on males. Non-pregnant males were significantly smaller than pregnant males in our sample (Fig. 4), so it is unlikely that all were collected at interbrood times (otherwise, they should be a random subsample of males). The small size of these non-pregnant males is consistent with both of the latter two possibilities mentioned above, so neither explanation can be rejected.

Female Mating Behavior

We detected four instances of multiple mating by females in our sample of 41 broods. Given the inherent limitations of sampling natural populations, the fact that we documented multiple mating at all suggests that dusky pipefish females

frequently have multiple mates. However, in the absence of information about population size, it is impossible to determine the precise frequency of multiple mating by females. Ideally, we would wish for a sample that included all breeding individuals at this locale because this would allow the direct documentation of successful matings by females. Failing that, we can estimate indirectly the proportion of the population sampled, and thereby assess the frequency of multiple mating by females.

Toward this end, the genotypic data on females can be viewed as providing information analogous to that employed in conventional mark/recapture studies to estimate local population size. The Lincoln-Peterson method is the simplest form of capture-recapture experiment (Pollock et al. 1990). First, a sample of n_1 animals is captured, marked and released. A second sample of n_2 animals is caught later and the number of marked animals in this sample (m_2) is recorded. Then, an estimate for the mean population size is given by (Pollock et al. 1990):

$$\hat{n} = (n_1 + 1)(n_2 + 1)/(m_2 + 1) - 1,$$

with variance

$$\widehat{\text{var}} \hat{n} = [(n_1 + 1)(n_2 + 1)(n_1 - m_2)(n_2 - m_2)] / [(m_2 + 1)^2(m_2 + 2)].$$

In our case, the original 'marks' are the inferred genotypes of mothers of the assayed broods. In effect, 37 females in this population marked themselves by depositing eggs in the males that were assayed. The matings probably occurred at least several days prior to our sampling because only more advanced embryos (those readily visible on the egg yolk) were assayed genetically. The 43 dusky pipefish females collected by seine represented our second or 'recapture' sample. Eleven of these females were 'marked' because they had contributed eggs to the broods of our pregnant males. Thus, in our case $n_1 = 37$ (the number of animals that were marked genetically), $n_2 = 43$ (the number of animals captured), and $m_2 = 11$ (the number of captured animals with the known genetic marks). Application of the Lincoln-Peterson formulas given above then yields an estimate of $n = 138$ (95% confidence interval 85–192) for the population size of adult dusky pipefish females at our microspatial locale. This number seems small, but may be plausible because pipefish are not considered highly vagile (Vincent et al. 1994). Of course, this estimate is not equivalent to N_e , the evolutionary effective population size, because the local breeding population represented in our sample may be part of a much larger metapopulation comprised of many such local breeding groups linked together by modest gene flow.

This model, of course, carries several assumptions (Pollock et al. 1990).

The population is closed. This assumption seldom is satisfied completely by any study, but its violation in our case would not change interpretations substantially. Any immigration to the population would be of unmarked individuals, and our estimate of population size would remain valid for the time of recapture (Pollock et al. 1990). Conversely, any deaths or emigrations that occurred at random with respect to marked and unmarked individuals would not affect the estimate of population size at the time of the mating events.

All animals are equally likely to be captured in all samples. If females remain near their mates after mating, population size could be seriously underestimated by this 'mark-recapture' approach. However, to affect our results appreciably, females would have to remain in proximity of their mated males until the embryos had developed to the stage assayed (probably several days to two weeks). Such mate fidelity has been reported for seahorses (Vincent and Sadler 1995), but in the pipefish *S. typhle* (the only member of the genus *Syngnathus* studied in this regard), females appear to move randomly through the seagrass habitat during the mating season (Vincent et al. 1994).

Marks are not lost or overlooked. This assumption is satisfied completely in the current application because individuals' genotypes are permanent, and because all of the adult females collected were genotyped. Thus, none of the genetic marks in our sample was overlooked.

The estimate of $n = 138$ females in the population, coupled with the observation of a 1:1 ratio of females to pregnant males (43 of each in our sample), yields an expected breeding population size of 276 adults (138 of each gender). Individual pregnant males carried an average of 1.86 broods (where a brood is defined here as a group of eggs from one mother and one father). So the total number of broods in the population at our time of sampling is expected to have been about 257, and as a first guess the mean number of mates per female may be $257/138 = 1.86$.

If we provisionally accept this (admittedly crude) estimate, models can be tested that assume random mating by females, such that female mating successes are either Poisson or binomially distributed (Sutherland 1985). Only one Poisson distribution is possible with mean (and variance) = 1.86, whereas any number of binomial distributions with the same mean but different variances can be envisioned. We tested the Poisson, and a series of 20 binomial distributions with variances ranging from 0.71 to 1.70. As the variance approaches the mean, binomial distributions become increasingly similar to the Poisson.

A series of computer simulations was carried out in which specified distributions of mating success were generated and populations of approximately 257 broods created, keeping track of the mother of each brood. In each of 10,000 replications for each simulated distribution, 41 broods (equivalent to our sample from nature) were sampled randomly without replacement. At the end of each simulation, broods in the computer sample were grouped into three categories: type 1 (those originating from mothers with only one brood represented in the sample of 41), type 2 (those from mothers with exactly two broods in the sample) and type 3 (those from mothers with more than two broods in the sample). By extracting random samples of 41 broods repeatedly (10,000 times), we generated expected numbers of the three types of broods for each frequency distribution of mating success under consideration. These expectations then were compared with empirical results from the genetic survey.

Recall that by genetic evidence the dusky pipefish sampled from nature contained 33 type 1 broods, eight type 2 broods, and zero type 3 broods. When evaluated against results of the computer simulations, neither the Poisson nor binomial distributions of female mating success could be rejected (χ^2 -

test, $\alpha = 0.05$) for these empirical data. Of course, other distributions might fit even better, but because the null model of random mating could not be rejected, such possibilities were not explored further. The best-fitting binomial distribution is presented in Figure 6B.

Although these simulations suggest that mating may be random for female dusky pipefish, the 11 females that mated with assayed males were significantly larger on average than other adult females in the sample. This suggests that larger females have a mating advantage and that mating is not random with respect to female quality, a finding also consistent with observations in other pipefish species that males prefer to mate with larger females (Berglund et al. 1986; Rosenqvist 1993). At least two explanations might account for the apparent discrepancy between these observations and the outcomes based on comparisons against the computer simulations. First, a binomial or Poisson distribution of female mating success need not be inconsistent with non-random mating with respect to intrinsic female quality, because the latter also may be distributed binomially (Arnold and Duvall 1994). Second, perhaps the available sample sizes simply provide insufficient statistical power to detect deviations from a binomial distribution of the magnitude that may be occurring in the study population.

Dusky Pipefish Mating System

The genetic mating system of this population of dusky pipefish is best described as polygynandrous (our definitions follow Searcy and Yasukawa 1995): both females and males frequently have multiple mates in the course of a single male pregnancy. There may exist temporal or spatial variation in this mating system not addressed by the current study because our sample is a point estimate from a single locale. The sex ratio at our study site was nearly 1:1, but observations at another site along the Gulf Coast indicate that sex ratios in this species can vary temporally (Brown 1972). Such variation could be important in terms of sexual selection intensity (Berglund 1994, 1995; Vincent et al. 1994), and might be an interesting topic for future investigation.

Detailed mating system information is available for two other species in the genus *Syngnathus*. Previous microsatellite analyses revealed a primarily polyandrous mating system in the Gulf pipefish *S. scovelli* (Jones and Avise 1997). Few males (< 5%) received eggs from more than one female, but females mated with multiple males in the time frame of a pregnancy. The causes of the mating system difference between *S. floridae* and *S. scovelli* are unknown, but a notable reproductive difference is the number of eggs carried per male: a mean of 50 to 60 by Gulf pipefish (Brown 1972; Jones and Avise 1997) versus 505 by dusky pipefish (current study). This difference stems from the smaller size of eggs in the dusky pipefish coupled with the larger size of males in this species (Brown 1972). If the evolution of a larger brooding capacity of dusky pipefish males has not been accompanied by a comparable increase in female fecundity, it would be difficult for a single female to fill a brood pouch to capacity. Other differences in the natural histories of the two species are related less obviously to reproduction, but may be of interest. Dusky pipefish are larger on average than

Gulf pipefish. Although the species occur in the same general habitats such that both were seined simultaneously, our experience suggests that Gulf pipefish had a higher population density at the study locale (assuming equal catchability).

Whereas the source of the difference in mating system between *S. floridae* and *S. scovelli* is unclear, the evolutionary consequences may be manifested as differences between the two species in patterns of sexual dimorphism. The Gulf pipefish is among the more sexually dimorphic members of the genus (Brown 1972; Dawson 1985), with females displaying silvery V-shaped stripes along the trunk, a deeply enlarged abdomen, and a large dorsal fin (Brown 1972). These characteristics might be interpreted as secondary sex characters. By contrast, dusky pipefish show little sexual dimorphism. Females have a slightly thicker abdomen than males and yellow coloration on the ventral surface, but these probably are primary sex differences resulting from the presence of ripe ova in adult females (Brown 1972). The comparisons are consistent with the hypothesis that a relationship should exist between the extent of polyandry and the extent of sexual dimorphism in sex role-reversed species (Jehl and Murray 1986). In this case, the more polyandrous Gulf pipefish displays a greater dimorphism in secondary sexual traits than does the less polyandrous dusky pipefish in which gender-based differences in the intensities of sexual selection presumably are less.

Extensive behavioral observations in the laboratory and field have established that the pipefish *S. typhle* also is polygynandrous (Berglund et al. 1988; Vincent et al. 1994). *S. typhle* has been described as sexually monomorphic (Berglund et al. 1986, 1989), but additional observations revealed that females display transient ornamentation during courtship (Vincent et al. 1992; Berglund and Rosenqvist 1993). In any event, both *S. typhle* and *S. floridae* appear to be less dimorphic in secondary sexual characters than *S. scovelli*. These observations again are consistent with the hypothesis that among sex role-reversed species the degree of polyandry should be related to the evolution of sexual dimorphism through sexual selection. To explore such hypotheses more fully, much additional information on the genetic mating systems of other syngnathid taxa will be required.

ACKNOWLEDGMENTS

Work was supported by a National Institutes of Health training grant to AGJ, and by a National Science Foundation grant and University of Georgia funds to JCA. We wish to thank G. Carney, G. Johns, and J. Behari for help with the collection effort and B. Nelson for assistance in the laboratory. M. Goodisman, G. Ortí, P. Prodöhl, and D. Walker provided valuable comments on the manuscript.

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Corresponding Editor: T. Markow