Genetic Evidence for Polygynandry in the Black-Striped Pipefish *Syngnathus abaster*: A Microsatellite-Based Parentage Analysis

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Abstract

Sexual selection theory predicts that, in organisms with reversed sex roles, more polyandrous species exhibit higher levels of sexual dimorphism. In the family Syngnathidae (pipefish, seahorses, and seadragons), males provide all parental care by carrying developing embryos on their ventral surfaces, and females develop secondary sex characters. Syngnathids exhibit a variety of genetic mating patterns, making them an ideal group to test predictions of sexual selection theory. Here, we describe the mating system of the black-striped pipefish *Syngnathus abaster*, using 4 highly variable microsatellites to analyze parentage of 102 embryos. Results revealed that 1) both sexes mate multiple times over the course of a pregnancy (polygynandrous mating system), 2) eggs are spatially segregated by maternity within each brood pouch, and 3) larger females have higher mating success (Kolmogorov–Smirnov test; $P < 0.05$). Together with similar studies of other syngnathid species, our results support the hypothesis that the mating system is related to the intensity of sexual dimorphism.

Key words: mating system, polyandry, sex-role reversal, sexual dimorphism, sexual selection, Syngnathidae

The study of mating systems has had a long-standing tradition in evolutionary biology and ecology. A wide range of studies has led to the general expectation that the mating system is closely related to the intensity of sexual selection and the degree of sexual dimorphism (Avise et al. 2002; Shuster and Wade 2003; Rosenqvist and Berglund 2011). Darwin (1871) suggested that, in organisms with conventional sex roles, more polygynous species show greater degrees of sexual dimorphism, as a result of an increased intensity in sexual selection. Analogous to Darwin's theory, it could be expected that, in organisms with reversed sex roles, more polyandrous species exhibit higher levels of sexual dimorphism (Jehl and Murray 1986). The study of mating systems in sex-role–reversed species, therefore, provides exceptional opportunities to test predictions of sexual selection theory (Williams 1975; Wilson et al. 2003).

Frequent occurrences of sex-role reversal can be found in the family Syngnathidae including pipefish, seahorses, and seadragons (Berglund and Rosenqvist 2003). These species are interesting because of male pregnancy, in which males provide all postzygotic care by brooding embryos on their ventral surfaces. The phenomenon of male pregnancy results in a higher investment by males than by females, and females must compete more than males for access to mates (Berglund and Rosenqvist 1993). Female length in particular seems to play an important role in the competition for access to mates (Ripley and Foran 2006; Paczolt and Jones 2010).

Microsatellite-based studies have revealed a wide range of genetic mating patterns in syngnathid fishes, making them an ideal group to investigate the relationship between the mating system and the degree of sexual dimorphism in sex-role–reversed species (Jones and Avise 2001). The sexually dimorphic gulf pipefish *Syngnathus scovelli* (Evermann and Kendall, 1896), for example, is characterized by a polyandrous mating system, in which males receive eggs from only 1 female but females mate with multiple males (Jones and Avise 1997a). Polygynandrous mating systems, in which both males and females mate multiple times over the course of a single pregnancy, have been reported for 2 pipefish species with intermediate levels of sexual dimorphism: the broad-nosed pipefish *Syngnathus typhle* (Linnaeus, 1758) and the dusky pipefish *Syngnathus floridae* (Jordan and Gilbert, 1882)

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(Jones and Avise 1997b; Jones et al. 1999). As in most seahorses, the narrow-bellied seahorse *Hippocampus subelongatus* Castelnau, 1873 displays little sexual dimorphism and is characterized by a monogamous mating system, in which 1 male mates solely with 1 female (Jones et al. 1998). To date, only polygyny has not yet been documented in the family Syngnathidae.

These previous studies support the hypothesis that in sex-role–reversed organisms, more polyandrous species show higher degrees of sexual dimorphism as a result of an increased intensity in sexual selection (Jones and Avise 2001). However, caution should be taken before broadly generalizing this pattern. The relation between mating systems, sexual selection, and sexual dimorphism is complicated, and additional descriptions of mating systems are needed to unravel its complexity (Rosenqvist and Berglund 2011).

In this study, microsatellite markers are employed to assess the mating system of a population of the black-striped pipefish *Syngnathus abaster* (Risso, 1827) from the Ria Formosa lagoon (South Portugal, Figure 1) where it is the fifth most abundant species (Erzini et al. 2002) and can easily be collected in shallow seagrass meadows. Previous studies

of *S. abaster* have focused mainly on its peculiar reproductive biology (Tomasini et al. 1991; Carcupino et al. 1997; Silva et al. 2009). Courtship behavior and modest levels of sexual dimorphism (females are slightly larger than males and show transient ornamentation patterns during mating season) suggest sex-role reversal in the black-striped pipefish (Silva et al. 2006). Visual observations of captive fish have shown that both sexes seek multiple mates during the course of a single pregnancy, suggesting a polygynandrous mating system (Silva et al. 2006). Genetic data on the natural mating system of *S. abaster*, however, are still lacking.

Under this framework, the main objective of the present study is to describe the mating system of *S. abaster*, using microsatellite-based parentage analysis. The primary aims are 1) to identify the number of females that contributed to each male's brood, 2) to assess the spatial distribution of full- and half-sib embryos inside the male's brood pouch, and 3) to document multiple mating of females. A secondary objective is to test for female sexual selection by assessing size differences between matched (females that are identified as mothers of the assayed embryos) and nonmatched females. The broader intent of the present study is to
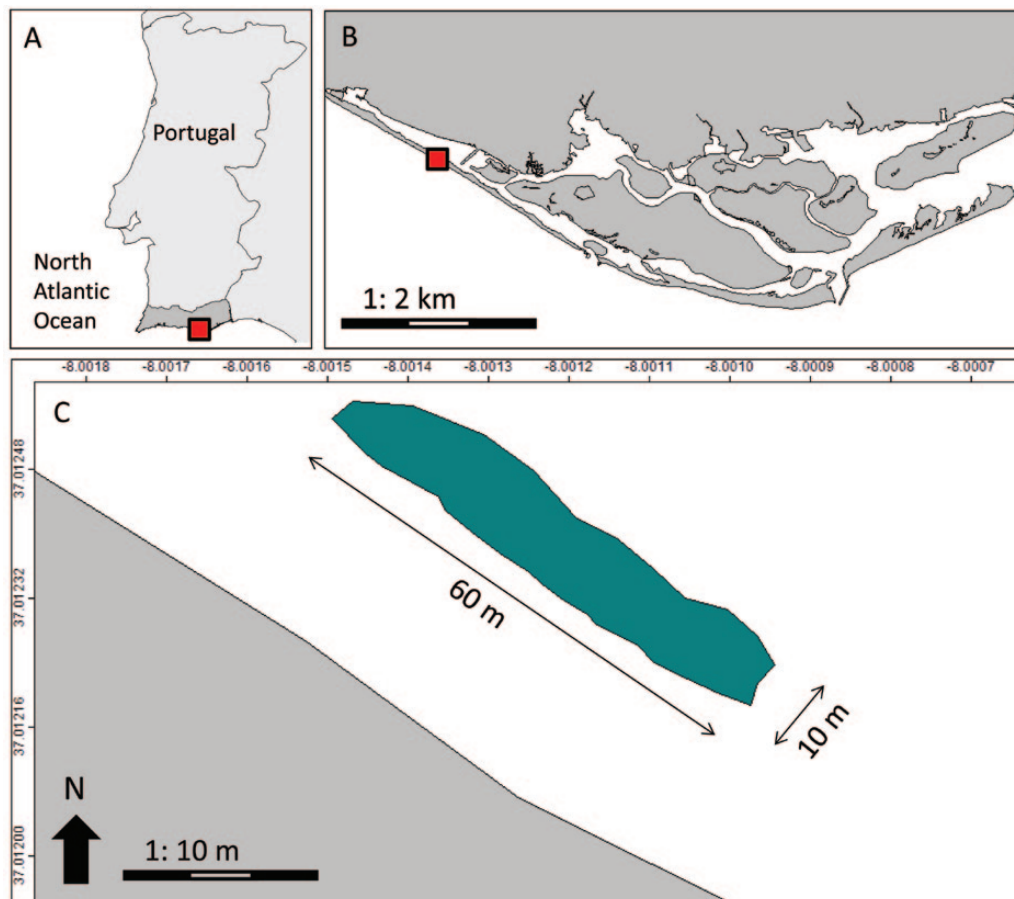

Figure 1. Map of southern Portugal (**A**), the western part of the Ria Formosa lagoon (**B**), and the location of the sampled seagrass patch, running parallel to the shoreline (**C**).

compare our results with previous studies of mating systems in syngnathids, addressing possible relationships between mating systems, sexual selection, and sexual dimorphism in sex-role–reversed species.

Materials and Methods

Collection of Field Samples

Pipefish were collected during two 5-day periods (12–16 March 2012 and 26–30 March 2012) from a shallow sea-grass bed in the Ria Formosa lagoon, located on the south coast of Portugal (36°59'N, 7°51'W) (Figure 1). Individuals were captured using a 7×1.60 m seine net of 5-mm mesh, which incorporated a central part (1 m length) of 3-mm mesh to enhance catching success. Pipefish identified as *S. abaster* were measured for total length to the nearest millimeter (L_T; from tip of snout to tip of caudal fin) and sexed. Males can be distinguished by the presence of a brood pouch located ventrally on the tail and females by distension of the anterior portion of the trunk. Juveniles do not exhibit these sexual characteristics. Genetic samples of females ($n = 74$) were obtained from a small piece of caudal fin and immediately placed in ethanol (99%). Pregnant males ($n = 21$) were taken to the laboratory in a container filled with seawater, sacrificed by placing the container into a freezer at -20 °C, and preserved in ethanol (99%). Nonpregnant males, females, and juveniles were released at the sampling site.

Microsatellite-Based Parentage Analysis

We dissected eggs ($n = 508$) from pregnant males, keeping track of their position within the brood pouch. The egg batch of each male was horizontally divided into 3 (up to 30 eggs) or 4 (up to 40 eggs) equal zones. Two eggs from each zone were randomly selected for genotyping ($n = 102$). Genomic DNA was isolated from fin clips (females and males) or eggs (embryos) following a protocol based on Sambrook et al. (1989) with minor modifications. To facilitate proteinase K digestion, eggs were cut into 4 pieces. Extracted DNA was quantified using a spectrophotometer (Thermo Scientific NanoDrop™ 1000). Individuals were genotyped at 4 microsatellite loci (28B3, 28E6, 28E8, and 28F3) previously isolated and characterized for *S. abaster* by Diekmann et al. (2009). PCR conditions for microsatellite amplification varied depending on the DNA yield from the sample (Appendix 1). PCR reactions were performed with the following program: an initial denaturation step of 5 min at 95 °C, 34 cycles consisting of 30 s at 94 °C, 30 s at a locus-specific annealing temperature (58 °C for 28B3, 60 °C for 28E6 and 28E8, and 63 °C for 28F3), 30 s at 72 °C, followed by a final extension step of 1 min at 72 °C. Fragment size was determined on an ABI prism 3130XL capillary sequencer using reverse primers labeled with FAM (Sigma-Aldrich, St Louis, MO) (28E6) or HEX (Sigma-Aldrich) (28B3, 28E8, and 28F3) and the GeneScan-500 LIZ size standard (Applied Biosystems, Inc., Carlsbad, CA).

Allele scoring was carried out with STRand 2.4.59 (http://www.vgl.ucdavis.edu/informatics/strand.php). Resulting raw allele sizes were binned using the R package MsatAllele (Alberto 2009).

Parentage assessment was conducted with CERVUS 3.0 (Kalinowski et al. 2007). Given the genotypes of the embryos, of their known fathers, and of the potential mothers, the maternity was assigned to the female with the largest log-likelihood ratio Logarithm Of Odds (LOD). The assumed error rate was 0.01, the average nonexclusion probability (second parent) for all loci combined was 0.00004, and the proportion of candidate mothers sampled was 0.07. The number of females contributing to an egg batch was determined considering the order of embryos inside the brood pouch, and the minimum number of mothers was estimated with GERUD 2.0 (Jones 2005). The expected exclusion probability was 0.9999, considering that 1 parent was known with certainty, 1 unknown, and including the 4 loci. The power of GERUD was assessed with GERUDsim 2.0 (Jones 2005). Parameters were specified according to the observed reproduction biology of *S. abaster*, as suggested in the manual. For 7 runs with 500 iterations each, the percentage of the correctly reconstructed number of mothers was estimated as follows: 26.8% (run 1), 84.8% (run 2), 0% (run 3), 53.8% (run 4), 97% (run 5), 72% (run 6), and 95.6% (run 7). Because most of the runs revealed large percentages, we assumed that the applied microsatellites had enough power for parentage analysis. The nonparametric Kolmogorov–Smirnov test was performed to determine differences in length between matched (females that were identified as mothers of the assayed embryos) and nonmatched females.

Allele frequencies, the number of alleles per locus, observed (H_o) and expected (H_e) heterozygosity were calculated using GENETIX 4.05.2 (Belkhir et al. 2004). For each locus, Hardy–Weinberg equilibrium (HWE) was tested by the exact test using the Markov chain method in ARLEQUIN 3.0 (Excoffier et al. 2005). Considering all loci together, HWE was tested by the chi-square test using GENEPOP on the web 4.0.10 (Raymond and Rousset 1995). Deviations from HWE were characterized by F_{IS} values (Weir and Cockerham 1984) with GENETIX. In instances where the observed genotype frequencies deviated significantly from HWE, the estimation of null allele frequencies, as the most probable cause of such HWE departures, was carried out using CERVUS.

Results

Microsatellite Markers

All 4 microsatellite loci were highly variable, which makes them extremely powerful for parentage assessment. The number of alleles per locus varied from 30 at 28E6 to 61 at 28B3 (Table 1). H_e averaged 0.958 across loci, ranging from 0.936 (28E6) to 0.976 (28B3). Significant deviation from HWE at a 0.05 α-level was detected only at locus 28B3 ($P < 0.001$). This deviation was characterized by a positive

F_{IS} value (F_{IS} = 0.099), indicating a deficit of heterozygotes. Heterozygote deficiencies may be the result of biological factors such as genetic drift or inbreeding, or an indicator of null alleles.

Parentage Analysis

All females (*n* = 74) and pregnant males (*n* = 21) caught during sampling were successfully genotyped at each of the 4 loci. Only 16 of the pregnant males carried embryos sufficiently developed for microsatellite assay. From these males, 102 embryos were collected and genotyped at all 4 loci. CERVUS assigned all embryos to their known father without any mismatches. Thus, there is complete confidence of paternity of the broods from each *S. abaster* male. Among all assayed embryos, 41 (40%) could be assigned to specific females that were collected during sampling at a confidence level of 95% (Figure 2). Assigned embryos matched at all 4 microsatellite loci. The remaining 61 embryos (60%) showed mismatches from candidate females at 2 or more loci. In total, 41 females contributed to the broods of pregnant males, of which 16 were caught during sampling.

Eggs were spatially segregated by maternity within each brood pouch. On average, males contained 24.19 ± 1.29 (standard error [SE]) eggs, ranging from 10 to 32 eggs. Each male received eggs from multiple females. The mean number of successful mates per male was 2.75 ± 0.19 (SE), ranging from 2 to 4 mates (Figure 3). The occurrence of embryos with identical mothers in the brood pouches of different males indicated that females mated with multiple males as

Table I Genetic variability in samples of adult black-striped pipefish at 4 microsatellite loci used for parentage analysis

Locus	Sample size	No. alleles	Range in allele size (bp)	H_o	H_e	Test of HWE (*P*)	F_{IS}
28B3	95	61	131–512	0.884	0.976	<0.001	0.099*
28E6	95	30	145–284	0.926	0.936	0.424[NS]	0.016[NS]
28E8	95	44	190–306	0.990	0.955	0.137[NS]	−0.031[NS]
28F3	95	44	226–466	0.958	0.965	0.139[NS]	0.013[NS]
All loci	95	179	—	0.940	0.958	<0.001	0.025*

F_{IS}, inbreeding coefficient; H_e, expected heterozygosity; H_o, observed heterozygosity; HWE, deviation from Hardy–Weinberg equilibrium; NS = not significant.
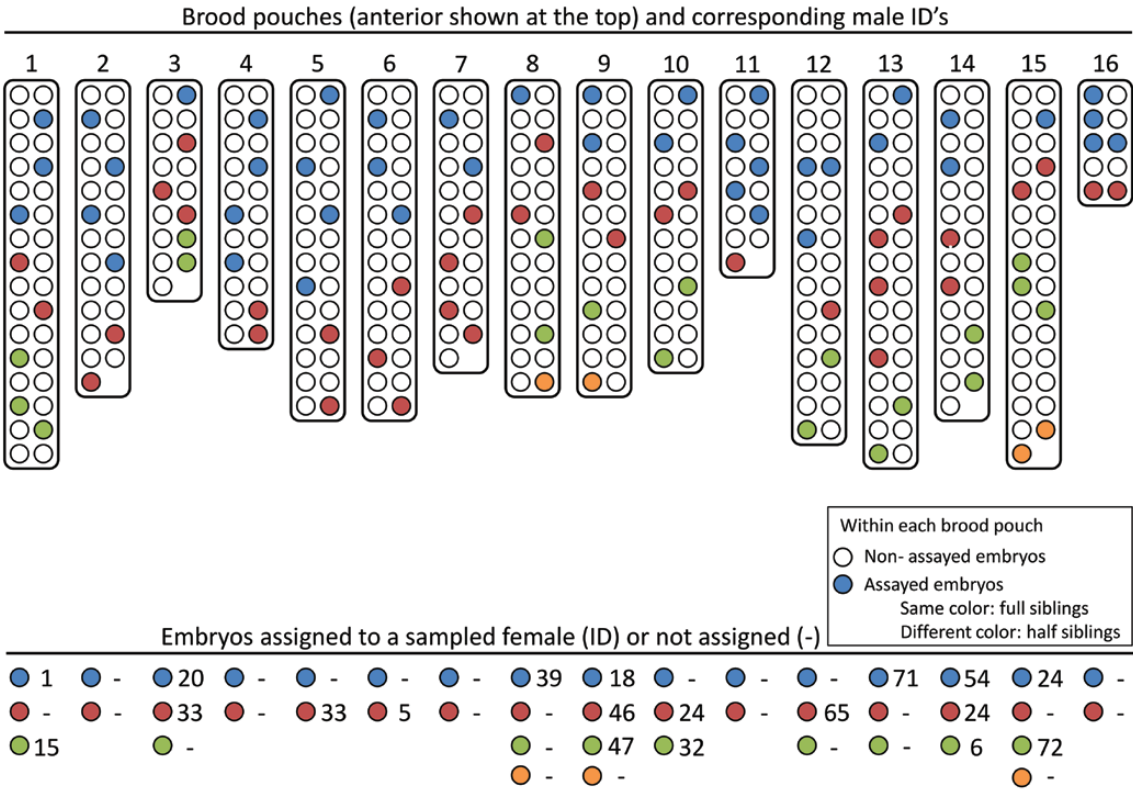*P < 0.001.

Figure 2. Spatial distribution of full- and half-sib embryos inside male brood pouches of *Syngnathus abaster*.
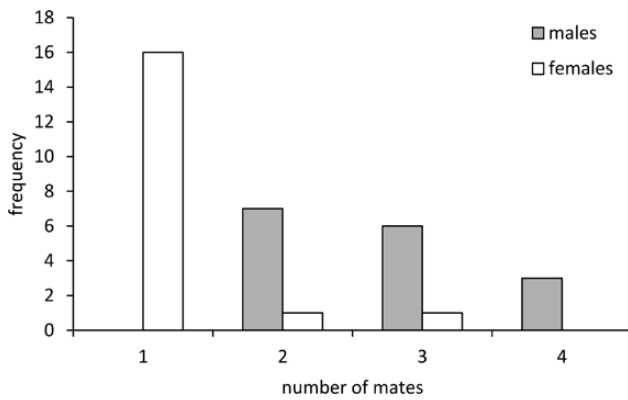
Figure 3. Mating success of male and female *Syngnathus abaster*.

well. One female (ID = 33) contributed to 2 broods, and another female (ID = 24) contributed to 3 broods (Figure 2). On average, matched females successfully mated with at least 1.19 ± 0.14 (SE) males.

To test for sexual selection, the total length of females identified as mothers of the assayed embryos (8.2 cm ± 0.13 SE) was compared with the total length of nonmatched females (7.5 cm ± 0.09 SE). Matched females were significantly larger than nonmatched females (Kolmogorov–Smirnov test; $P < 0.05$). Pregnant males exhibited a mean total length of 8.4 cm ± 0.14 (SE), ranging from 7.0 to 12.2 cm.

Discussion

This study demonstrated a polygynandrous mating system in a sex-role–reversed species, *S. abaster*. It also provided information on the reproductive biology of this species, namely showing variance in female reproductive success that depends on a morphological trait, female size.

Microsatellites and Parentage Analysis

Four highly polymorphic microsatellite markers were used to assign maternity. Previous parentage studies on syngnathids successfully used between 2 and 4 microsatellites (Jones et al. 1999; Mobley and Jones 2009; Wilson 2009). Maternity was assigned with high confidence indicating that markers were sufficiently powerful to reliably identify true mother–offspring relationships. Nonamplifying null alleles can potentially complicate microsatellite-based parentage analysis (Pemberton et al. 1995). However, the apparent presence of null alleles at locus 28B3 should not compromise parentage analysis. CERVUS treats mismatches caused by null alleles as if they are genotyping errors and can assign maternity on the basis of the other 3 loci (Kalinowski et al. 2007). The reasons given above indicate that maternity was accurately assigned.

Reproduction Biology and Mating System of *S. abaster*

Pregnant *S. abaster* males carried an average of 24 eggs. This number seems small in comparison with the results of

a previous study, which reported a mean clutch size of 37 eggs (Silva et al. 2006). Two factors apparently influence the number of eggs inside a male's brood pouch: the male's total length (Silva et al. 2008; Mobley and Jones 2009) and the timing of the breeding season (Riccato et al. 2003). Mean clutch size increases to a peak in the middle and decreases toward the end of breeding season. Given that pregnant males studied by Silva et al. (2006) exhibited lengths similar to those of our pregnant males, the observed small clutch size may rather be explained by the sampling dates at the beginning of the breading season. Thus, the number of eggs carried by *S. abaster* males is expected to further increase toward the middle of the breeding season.

Each male received eggs from multiple females during the course of a single pregnancy. The mean number of successful mates per male was 2.8 ranging from 2 to 4 females. Previous genetic analysis revealed a lower level of multiple maternity in *S. floridae* (Jones and Avise 1997a) but a much higher rate of multiple mating in *S. typhle* males (Jones et al. 1999). Reasons for differences in the rate of multiple maternity may be various and are probably to be found in ecological factors, such as adult sex ratio, population density, and temporal availability of mates, all of which affect these species differentially (Shuster and Wade 2003; Kokko and Rankin 2006; Mobley and Jones 2009). Eggs were spatially segregated by maternity within each brood pouch. This result can be explained by the specific mating behavior of *S. abaster*. During copulation, the female deposits unfertilized eggs through an opening at the top of the male's brood pouch. Immediately after egg transfer, males release sperm directly into the pouch (Dzyuba et al. 2008) and perform body contractions that cause packing of the eggs in the posterior end of the marsupium (Silva et al. 2006). Embryos from the first mating partner are therefore found at the pouch bottom, with eggs of successive mating partners stacked successively on top. A spatial segregation of eggs by maternity was also shown in *S. typhle* and *S. floridae* (Jones and Avise 1997b; Jones et al. 1999).

Two females contributed to more than 1 brood, providing evidence for multiple mating of *S. abaster* females. However, the true rate of multiple mating by females is probably underestimated as the population was not sampled exhaustively, and females may have mated with additional males that were not collected. The undersampling of pregnant males seems to be small though, because individuals were shown to have equal catchability (Chapman's test; $P = 0.1103$) and the observed sex ratio (0.9:1) was consistent with previous findings (Tomasini et al. 1991; Franzoi et al. 1993). However, given this potential sampling bias, the fact that multiple mating by females was discovered at all indicates that *S. abaster* females commonly mate with several males. Thus, the mating system of the black-striped pipefish can be described as polygynandrous with multiple mating by both sexes over the course of a single pregnancy. Although a polygynandrous mating system of *S. abaster* was previously reported (Silva et al. 2006), this is the first study presenting molecular evidence for multiple mating of *S. abaster* males and females.

According to the theory of Trivers (1972), the sex with the largest investment becomes a limiting resource, causing the members of the other sex to compete for mating opportunities. In most animals, males must compete more than females for access to mates. In the family Syngnathidae, however, the phenomenon of male pregnancy results in a higher investment by males instead of females. The usual direction of sexual selection is therefore reversed, such that females compete more intensively for access to mates (Berglund and Rosenqvist 1993). As is shown in this study, female length seems to play an especially important role in the competition for access to mates. Matched females (females that are identified as mothers of the assayed embryos) were significantly larger than nonmatched females, suggesting that larger females outcompete smaller females for matings or that males prefer to mate with larger females. Preference of larger females was also shown for *Syngnathus fuscus* (Storer, 1839) and *S. floridae* (Ripley and Foran 2006). A study of mate choice in *S. scovelli* additionally showed that the elapsed time before mating took place was significantly shorter for larger females (Paczolt and Jones 2010). Furthermore, that study found that female size was positively correlated with the number of eggs transferred to the male's pouch and with the number of viable offspring. Thus, larger females seem to confer more fitness than smaller females. By mating with large females, males are expected to benefit from enhanced offspring survivorship. Large females of *S. abaster* produce large eggs (Silva et al. 2008). Offspring hatching from larger eggs have been reported to have higher survival, higher resistance to starvation, and increased swimming performance (Kolm and Ahnesjo 2005).

Syngnathid Mating Systems and Sexual Selection Theory

A wide range of mating systems have been documented in the family Syngnathidae, including monogamy in *Hippocampus abdominalis* (Lesson, 1827) and *Hippocampus angustus* (Günther, 1870) (Jones et al. 1998; Wilson and Martin-Smith 2007), polyandry in *S. scovelli* (Evermann and Kendall, 1896) and *Nerophis ophidion* (Linnaeus, 1758) (Jones and Avise 1997a; McCoy et al. 2001), and polygynandry in *Syngnathus leptorhynchus* (Girard, 1854), *S. floridae*, and *S. typhle* (Jones and Avise 1997b; Jones et al. 1999; Wilson 2009). In sex-role–reversed species, sexual selection and sexual dimorphism should be most extreme in polyandrous species, intermediate in polygynandrous, and least extreme in monogamous species (Jones and Avise 2001; Rosenqvist and Berglund 2011). In the polyandrous pipefish *N. ophidion*, for example, females have intense blue ornaments and are much larger than males (Rosenqvist 1990). By contrast, the polygynandrous pipefish *S. typhle* exhibits only transient female ornamentation during courtship and reduced levels of sexual size dimorphism (Berglund and Rosenqvist 1993). The monogamous seahorse *H. subelongatus*, finally, is sexually monomorphic (Jones et al. 1998). These and additional studies of syngnathid species reveal a trend generally consistent with sexual selection theory, predicting higher levels of sexual dimorphism in sex-role–reversed polyandrous species (Jones and Avise 2001; Rosenqvist and Berglund 2011). Results of the present study further support the hypothesis because *S. abaster* displays intermediate levels of sexual dimorphism (females show transient coloration during mating season and are slightly larger than males; Silva et al. 2009) and exhibits a polygyandrous mating system.

However, the relationships between mating system, intensity of sexual selection, and degree of sexual dimorphism are very complex and are affected by various environmental, temporal, and demographic factors (Shuster and Wade 2003; Mobley and Jones 2009). Future studies on mating systems, especially of other syngnathid taxa and sex-role–reversed species outside the family Syngnathidae, would be highly valuable to gain a deeper understanding in sexual selection theory and the evolution of mating systems.

Funding

Acknowledgments

Appendix 1 PCR conditions for microsatellite amplification

	DNA concentration [ng/μL]		
	>10	5–10	<5
Reaction volume [μL]	10	10	15
Template DNA [μL]	1	2	4
$MgCl_2$ [mM]	2	2	2
Primer (forward and reverse) [μM]	1	1	1
Each dNTP [μM]	2	2	2
GoTaq Flexi buffer (Promega)	10×	10×	10×
GoTaq DNA Polymerase (Promega) [U]	2,5	2,5	6

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