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## A MICROSATELLITE ASSESSMENT OF SNEAKED FERTILIZATIONS AND EGG THIEVERY IN THE FIFTEENSPINE STICKLEBACK

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**Abstract.**—Attempts by males to steal fertilizations from other males are common in many species. In some sticklebacks, males also are known to steal eggs from the nests of rivals and to carry them back to their own nests. However, the genetic consequences of these nest-raiding behaviors seldom have been investigated. Here we assess genetically the prevalence of sneaked fertilizations and egg stealing, and we describe the mating system in a natural population of the fifteen-spine stickleback. Six microsatellite markers were developed and employed to assay a total of 1307 embryos from 28 nests. Guardian males and all nest-holding males in the local area also were genotyped for two to six loci. Analysis of male genotypes and those of embryos revealed that five of the 28 nests (18%) contained progeny from sneaked fertilizations, and that four of the 24 nests (17%) with resident males contained stolen egg clutches. Comparisons of the composite DNA genotypes of nest-holding males against those of inferred sneakers implicated one nest holder as the sneaker of a nest seven meters from his own. Also, the genetic data demonstrated that nests of males frequently contain eggs from multiple females. The multilocus genotypes of inferred mothers indicated that females mate with multiple males, sometimes over distances greater than one kilometer.

**Key words.**—Cuckoldry, kleptogamy, mating system, polygamy.

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Among the varied reproductive tactics adopted by animals, many are “parasitic” in that they exploit the reproductive efforts of rivals. Perhaps the best-known examples include extrapair copulations (EPC) in birds (Birkhead and Møller 1992) as well as sneaky copulations by satellite or subordinate males in mammals, reptiles, fishes, and other taxa (e.g., Arak 1988; Koprowski 1993; Ohsawa et al. 1993; Brockman et al. 1994; Taborski 1994; Sinervo and Lively 1996; Wikelski et al. 1996).

In the case of EPC, or sneaking, the parasitic male may steal fertilizations from other males who have gained access to females. The sneaker may profit in at least two ways. First, by mating covertly or forcibly with females attracted by another male, a sneaker may achieve fertilizations without the added energy expenditure necessary to compete for mates (either intra- or intersexually; e.g., Hutchings and Myers 1988; Marconato and Shapiro 1996; Wikelski et al. 1996). Second, in taxa with male parental care, a successful sneaker may parasitize a rival male’s parental efforts as well (e.g., Gross 1979).

The costs to parasitized individuals of lost fertilizations and of counterproductive investment in another’s offspring should produce strong selection pressures to evolve defenses against sneaker males. Conversely, the benefits to sneakers of gained fertilizations and of parental services by other males should produce strong selection pressures favoring parasitic reproductive behavior. Thus, it is unclear what, if any, balance may be achieved in the frequency of reproductive parasitism (Barnard and Sibly 1981). Nevertheless, field and laboratory observations reveal that sneaky reproductive behavior is a widespread phenomenon in many taxa. Many other less-common parasitic reproductive strategies, such as brood parasitism in birds (Petrie and Møller 1991), also have been documented. As a group, teleost fishes have perhaps the most bewildering array of parasitic reproductive modes (reviewed in Taborsky 1994).

In addition to numerous other tactics, male fishes sometimes exploit rivals by the theft of nests built by other males (Bisazza and Marconato 1988; Unger and Sargent 1988; Bisazza et al. 1989) and by the theft of eggs from other males’ nests (Wootton 1971; Mori 1995). The former offers obvious potential advantages to the thief, but the reproductive benefit to egg thievery is uncertain. One hypothesis is that egg-stealing males have enhanced fitness because females may prefer to spawn with males whose nests already contain eggs (Ridley and Rechten 1981; Sikkell 1989; but see Jamieson and Colgan 1989). Another possibility is that the extra eggs may provide a predation dilution effect (Whoriskey and Fitz-Gerald 1994).

Sticklebacks are among the most intensively studied groups of fish with regard to reproductive behavior (e.g., Wootton 1984; Bell and Foster 1994). The threespine stickleback (*Gasterosteus aculeatus*) has been a workhorse of fish behavioral ecology, and males of this species engage in numerous parasitic tactics (Mori 1995). Most common in *G. aculeatus* are sneaky fertilizations and egg thievery, behaviors often grouped under the collective term “nest-raiding” (Li and Owings 1978).

In all sticklebacks, males use glue secreted from the kidney (Hentschel 1979) to build nests in the substrate or in vegetation (Morris 1952; Rowland 1994; Wilmott and Foster 1995). The nest builder attracts females to the nest. After courtship rituals of varied complexity among species, eggs are laid inside the nest. The male then swims through the nest and releases sperm. Occasionally, a second male (a sneaker) approaches during this courtship and quickly passes through the nest releasing sperm (Morris 1952; van den Assem 1967). Egg stealing also occurs in sticklebacks, facilitated by a clumping together of eggs within a nest into distinct, portable clusters that can be stolen *en masse* (Mori 1995). The female stickleback provides no parental care whereas the guardian male fans, guards, cleans, and repairs

the nest until the progeny hatch (Potts et al. 1988; Östlund 1995).

Research on sneaking behavior in the laboratory or nature traditionally has involved visual observations that provide no information on the fertilization success rate of sneakers. Although molecular markers offer great power for genetic parentage analyses (reviews in Birkhead and Møller 1992; Avise 1994; Westneat and Webster 1994), only a few studies have applied hypervariable genetic markers to questions of maternity and paternity in fishes (Kellogg et al. 1995; Colbourne et al. 1996; Parker and Kornfield 1996; Jones and Avise 1997a,b). In one study of special relevance here, Rico et al. (1992) used DNA fingerprinting to detect sneaking and egg thievery in a natural population of threespine sticklebacks. Here we employ a battery of microsatellite markers to carry out a more extensive analysis of the mating behavior of a related species, the fifteen-spine stickleback, *Spinachia spinachia*.

Our goals in this study were to develop and use microsatellite markers to (1) document the frequency of sneaked fertilizations and egg thievery by males in the wild; (2) estimate the frequency of concurrent multiple mating by males and the numbers of female broods cared for simultaneously by individual males; (3) investigate the identity of the sneaker males; (4) document multiple mating by females; and (5) place all of these findings in a spatial context by analyzing map positions of the collected nests.

## MATERIALS AND METHODS

### *Collection and Treatment of Field Samples*

Males and their nests were collected during May and June 1996 from the Gullmar Fjord near Klubban Biological Station on the Swedish West Coast (58°15'N, 11°28'E). Individual nests were located by snorkeling at depths of one to two meters, and any male found closely associated with a nest was collected together with his nest. Males and their nests (usually containing progeny) were returned live to the laboratory, where clutches were weighed, males measured, and samples of embryo-containing eggs frozen for microsatellite analysis. Specimens packed on dry ice were taken to the University of Georgia for genetic assay.

Tissue was prepared for PCR following Jones and Avise (1997a). Individual embryos were separated from one another under a dissecting scope and removed from the outer egg shell. Yolk was rinsed away in deionized water and embryos were placed individually in microcentrifuge tubes with 50–150  $\mu$ L of Gloor and Engels' (1992) fly buffer. Samples then were incubated at 37°C for 30 min followed by 2 min at 95°C, and spun at high speed for 2 min in a microcentrifuge; 2  $\mu$ L of the resulting supernatant was used as a template for PCR. A similar approach was used to prepare tissue from adults using a small caudal fin clip.

### *Microsatellite Assays*

To identify suitable markers for *S. spinachia*, we first amplified microsatellite loci using PCR primers developed previously for the threespine stickleback (Rico et al. 1993). None of these loci proved polymorphic in our samples of fifteen-

spine sticklebacks. Thus, we cloned microsatellite loci specifically from *S. spinachia* as follows.

Total genomic DNA was isolated from a single specimen of *S. spinachia* using a standard proteinase K, phenol:chloroform extraction procedure. The DNA was digested with *Mbo*I and the 200–700-bp fragments were ligated into *Bam*HI digested, dephosphorylated pBluescript phagemid (Stratagene). Ligations were transformed into competent XL1-Blue *E. coli* (Stratagene). The resulting partial genomic library was screened first with a cocktail of the synthetic oligonucleotides (GT)<sub>10</sub>, (GGAT)<sub>4</sub>, (GACA)<sub>4</sub>, and (TAG)<sub>6</sub>, followed by a second cocktail of (GATA)<sub>4</sub>, (GA)<sub>10</sub>, (TCC)<sub>5</sub>, and (TTAGGG)<sub>3</sub>. Of approximately 500 colonies screened, 21 hybridized to one or more of the probes. All positives were sequenced using the *fmol* DNA Sequencing System (Promega), and primers were designed to amplify the microsatellite repeats for six loci.

Microsatellites were amplified, after end-labeling one of the primers with 1  $\mu$ Ci  $\gamma$ -<sup>32</sup>P ATP per 5 pmol of primer, by performing PCR on the collected specimens. The PCR conditions consisted of 10  $\mu$ L reaction volumes containing 1 $\times$  Promega *Taq* buffer, 1.5 mM MgCl<sub>2</sub>, 0.15  $\mu$ M of each primer, 0.1 mM of each dNTP, and 0.5 units of Promega *Taq* polymerase. These reactions were placed in a Perkin-Elmer thermal cycler for an initial denaturation of 2 min at 94°C, followed by 30 cycles of 94°C for 1 min, an optimal annealing temperature for 1 min, and 72°C for 1 min. A final extension of 4 min at 72°C concluded the thermal cycling profile. For loci *Fifsp1* and *Fifsp3*, the optimal annealing temperature was 60°C, whereas for *Fifsp5* and *Fifsp15* the optimal annealing temperatures were 56°C and 54°C, respectively. The loci *Fifsp10* and *Fifsp16* were multiplexed by adding the second set of primers to the standard PCR cocktail and changing the thermal cycling parameters to include 8 cycles of 94°C for 1 min and 68°C for 1 min, followed by 25 cycles of the standard cycling parameters above with an annealing temperature of 64°C. The radioactive PCR products then were resolved on standard 6% polyacrylamide denaturing sequencing gels followed by overnight autoradiography.

### *Sampling Design*

Forty-six nests with males were collected, plus several nests without resident males. Five nests did not contain eggs, and of the remaining nests, 28 contained progeny sufficiently developed for microsatellite analysis. Of these 28 nests, 24 had guardian males whereas four were not associated with a male. All males in our collection plus 30–90 embryos from each of the 28 nests were assayed for two loci (*Fifsp10* and *Fifsp16*). Within some nests, up to three distinct clutches were visible as spatially segregated egg masses. We assayed a total of 1307 embryos from 44 clutches, an average of 30 embryos from each distinct clutch per nest. This number was chosen because it provides a reasonably high probability of detecting modest contributions of eggs by multiple females and of successful fertilizations by a sneaking male. For example, if a sneaker fertilized 10% of the eggs in a clutch, the binomial probability is > 95% that a random sample of 30 eggs from that clutch would include at least one of these sneaked-fertilization eggs. For the nests in which sneaking was deduced

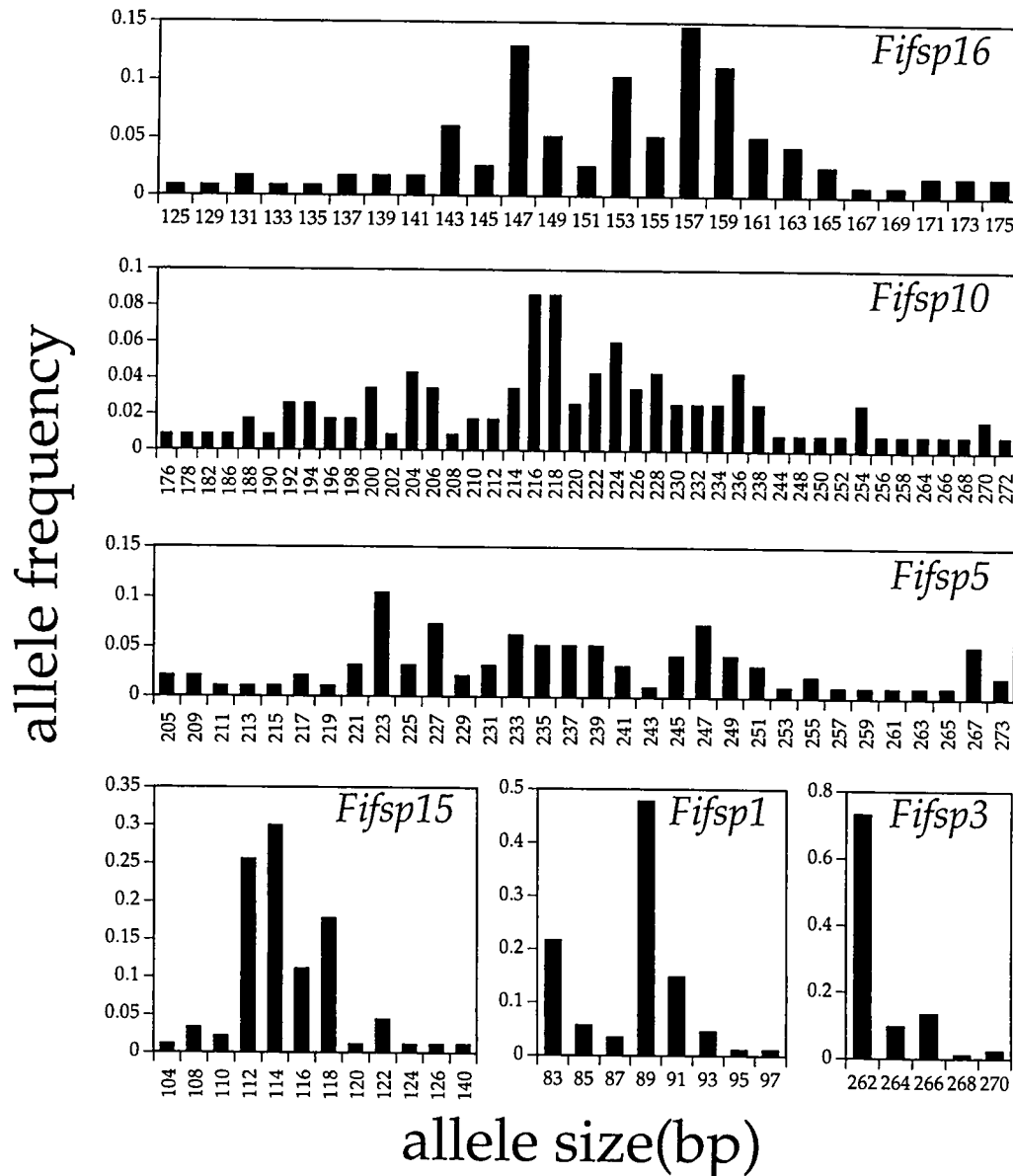


FIG. 1. Allele frequency histograms for the six microsatellite loci in the collection of adult male *Spinachia spinachia*.

(and in other special cases, see below), additional microsatellite loci were used to gain further genetic resolving power and to establish multilocus profiles for individuals of special interest.

## RESULTS

### *The Microsatellite Loci*

The six microsatellite loci for which primers were designed displayed 5–42 alleles each (Fig. 1, Table 1). Observed and expected heterozygosities ranged from 0.44 to 1.00. No locus deviated significantly from Hardy-Weinberg equilibrium in the wild-caught adult sample (exact test of Guo and Thompson [1992] as implemented in GENEPOP [Raymond and Rousset 1995]).

Of the 15 possible tests of linkage disequilibrium between

pairs of loci in the adult population (exact test in GENEPOP), only one outcome was significant (involving loci *Fifsp1* and *Fifsp15*;  $P = 0.026$ ). Given this number of tests, one significant result might be expected under the null hypothesis of independent assortment for the loci examined. Further tests for linkage of *Fifsp1* with *Fifsp15* involved examination of genotypic combinations within progeny arrays. No evidence of linkage was detected by this criterion (contingency  $\chi^2$ -tests,  $P > 0.05$ ).

To facilitate comparison against other published estimates of exclusionary power in genetic parentage analyses, conventional exclusion probabilities (the expected proportion of unrelated males that can be excluded as the father given a mother-offspring pair; Chakraborty et al. 1988) are provided in Table 1 for each locus. The combined exclusion probability for all six loci is extremely high (0.9998), with the majority

TABLE 1. Summary of the six *Spinachia spinachia* microsatellite loci. Shown are the forward and reverse primer sequences, microsatellite motif of the original cloned sequence, number of alleles observed in a random sample of  $n$  wild-caught males, and observed and expected heterozygosities. Also shown is the average exclusion probability (i.e., the expected proportion of unrelated males excluded as the father of a randomly chosen mother-offspring pair; see text) for each locus.

Locus	Primer sequences 5' → 3'	Cloned repeat	No. of alleles	$n$	Heterozygosity		Excl. prob.
					obs.	exp.	
<i>Fifsp16</i>	CTTTTCTGCCGGGGTTTCTTAT GTCGGCGGCTCACATTGAC	[TG] <sub>31</sub>	25	58	0.914	0.928	0.841
<i>Fifsp10</i>	CCCCAAGCCTCTCTCAAACACC ATGCTGCCGCTGAACCTTTGAC	[CA] <sub>25</sub>	42	58	0.948	0.969	0.921
<i>Fifsp5</i>	TCCGAGAGTCGCTTTAATCTT GTTACAGCCATTTATTGAACATC	[TG] <sub>34</sub>	30	48	1.000	0.962	0.903
<i>Fifsp1</i>	TCATGCAGATGTGTGCTAACTC CTTTGCGAGACACTTTTAACAA	[CA] <sub>16</sub>	8	44	0.682	0.705	0.477
<i>Fifsp3</i>	CATGGAGGAGACGTTGACTG CAGCAATCATTTCATTTCTGTAA	[CA] <sub>14</sub>	5	41	0.463	0.442	0.245
<i>Fifsp15</i>	GGAGGGAAAACCTGTCACAA GTGAGCTTTCTTTACGTATT	[AC] <sub>21</sub>	12	45	0.867	0.805	0.608

of resolving power attributable to the three most polymorphic loci.

Two sources of error for microsatellite loci must be considered in parentage analyses. False exclusions can result either from mutations or null alleles. In studies involving progeny arrays, null alleles should be evident from parents

TABLE 2. Genotypes of nest-guarding males collected from a natural population of fifteen-spine sticklebacks for the loci *Fifsp10* and *Fifsp16*. Also shown are the exclusion probabilities and probabilities of identity based on these two loci. In this case, the exclusion probability represents the expected proportion of offspring for which the resident male would be excluded as a potential father given a single unrelated embryo for whom *neither* parent is known with certainty. It is important to realize that this value is not the same as (and is less than) the exclusion probability for a male given a mother-offspring pair (Table 1). The probability of identity is simply the expected frequency of the male's genotype in the population under Hardy-Weinberg assumptions.

Male	Genotype for locus		Exclusion probability	Probability of identity
	<i>Fifsp16</i>	<i>Fifsp10</i>		
F1	147/147	230/270	0.980	$1.5 \times 10^{-5}$
F2	147/147	218/254	0.949	$7.5 \times 10^{-5}$
F4	147/161	194/236	0.956	$3.0 \times 10^{-5}$
F8	135/173	200/248	0.996	$1.8 \times 10^{-7}$
F24	147/161	190/218	0.940	$2.0 \times 10^{-5}$
F37	157/163	216/222	0.917	$9.4 \times 10^{-5}$
F41	143/145	200/220	0.981	$5.6 \times 10^{-6}$
F42	153/159	206/218	0.913	$1.4 \times 10^{-4}$
F44	159/165	234/256	0.983	$2.6 \times 10^{-6}$
F45	133/153	228/238	0.972	$3.4 \times 10^{-6}$
F46	155/157	188/224	0.947	$3.2 \times 10^{-5}$
F47	147/153	216/222	0.900	$2.0 \times 10^{-4}$
F48	137/143	224/238	0.975	$6.5 \times 10^{-5}$
F51	131/157	218/238	0.937	$2.3 \times 10^{-5}$
F53	145/155	224/254	0.975	$8.4 \times 10^{-6}$
F54	147/165	216/232	0.939	$3.0 \times 10^{-5}$
F55	153/155	188/216	0.944	$3.2 \times 10^{-5}$
F61	145/159	178/202	0.991	$8.6 \times 10^{-7}$
F62	147/173	258/272	0.991	$6.6 \times 10^{-7}$
F63	147/159	222/270	0.950	$4.3 \times 10^{-5}$
F65	149/159	198/216	0.941	$8.0 \times 10^{-6}$
F66	157/161	228/230	0.947	$4.5 \times 10^{-5}$
F68	125/163	226/236	0.985	$2.2 \times 10^{-6}$
F69	157/163	216/228	0.924	$9.4 \times 10^{-5}$

who appear homozygous but fail to transmit the observable allele to some progeny. In our case, signatures of null alleles were not detected, either by deviations from Hardy-Weinberg equilibrium or as non-Mendelian segregation of genes in the progeny arrays. Indeed, at *Fifsp10*, no nest-holding male was homozygous (so no null allele could be present) and only two nest-holding males appeared homozygous at *Fifsp16* (Table 2).

A second potential source of error involves *de novo* mutations that could cause a false exclusion. To circumvent this complication, we considered an exclusion valid only if it could be confirmed by two or more loci or if it involved an entire progeny array (i.e., if the male shared no allele at a locus with any of the brood). Mutation seems not to have been a problem in the current study, however, because all apparent single-locus exclusions that involved only a few embryos were confirmed by data from additional loci.

### Male Sneaking Behavior

In our initial analysis, we considered a nest to have been subjected to sneaked fertilizations if a single male (usually the resident guardian) was excluded as the father of some but not all of the embryos within at least one clutch from the nest. In other words, sneaking is evidenced by multiple paternity within clutches. By this definition, 5 of the 28 nests (18%) contained progeny resulting from sneaked fertilizations (Table 3). Given the fact that the genetic exclusions are absolute (barring mutation or null alleles; see above), this represents a minimum estimate of the frequency of sneaking in this population of *S. spinachia*. Table 4 includes a clutch-by-clutch breakdown of the percentages of eggs fertilized by sneaker males. For clutches in which sneaking had occurred, these ranged from 23% to 63%.

One of the sneaked nests (N4; Table 3) was collected without a resident male. Sneaking in this case was deduced because for all three loci for which the 30 embryos were assayed, the clutch could not have had a single parent of either gender. For all three loci, three distinct and nonoverlapping classes of heterozygotes were observed in the progeny. For example, for *Fifsp16* we detected the genotypes 153/159, 139/

TABLE 3. Nest-holding males assayed (F1–F69) and nests collected without a resident male (N1–N50). Map locales refer to Figure 3. Shown are dates of collection, male length, the number of distinct clutches in each nest (see text) with the number of clutches assayed in parentheses, the number of females whose eggs appeared within each male's nest, and the numbers of eggs (embryos) collected and assayed from each nest. If the male's nest contained hatched fry, the number of eggs is left blank (many of the fry escaped during collection or were otherwise missing). The last column indicates whether the male's nest contained eggs that were inferred to have been stolen or sneaked based on the microsatellite data.

Male	Date caught	Map locale	Length (mm)	No. of clutches	No. of mates	No. of eggs	No. eggs assayed	Stolen or sneaked?
F1	5/14/96	2	107	2 (2)	2	1503	60	sneaked
F2	5/14/96	2	132	3 (2)	0	1029	60	stolen
F4	5/14/96	2	117	1 (1)	1	366	30	no
F8	5/12/96	1	115	3 (3)	5	1753	90	sneaked
F24	5/26/96	3	116	1 (1)	1	255	30	no
F37	6/16/96	2	125	2 (2)	0	310	62	stolen
F41	6/16/96	2	120	2 (2)	4	1559	60	no
F42	6/16/96	6	122	2 (2)	5	1948	60	no
F44	6/16/96	2	108	3 (3)	7	—	85	no
F45	6/16/96	6	115	1 (1)	3	—	30	sneaked
F46	6/16/96	6	118	2 (2)	1	653	39	stolen
F47	6/20/96	3	122	2 (2)	2	814	76	no
F48	6/20/96	3	119	1 (1)	2	—	40	no
F51	6/20/96	3	106	3 (3)	4	1099	85	sneaked
F53	6/20/96	3	113	1 (1)	5	—	32	no
F54	6/19/96	2	—	1 (1)	1	254	39	no
F55	6/25/96	2	123	1 (1)	1	481	72	no
F61	6/26/96	2	128	2 (2)	2	367	30	no
F62	6/26/96	2	132	2 (2)	2	697	30	no
F63	6/26/96	2	119	1 (1)	1	567	30	no
F65	6/28/96	4	127	1 (1)	4	—	34	no
F66	6/29/96	1	120	1 (1)	0	582	30	stolen
F68	7/04/96	1	122	1 (1)	2	246	30	no
F69	7/04/96	1	96	1 (1)	3	—	30	no
N1	5/14/96	7	—	1 (1)	1	1163	30	—
N2	5/14/96	7	—	3 (1)	1	1320	30	—
N4	5/12/96	1	—	2 (1)	2	706	30	sneaked
N50	6/20/96	3	—	2 (2)	2	556	53	—

163, and 137/147 among the progeny, indicating that at least three paternal alleles were present. By the same logic, this clutch had multiple mothers also. The *largest* number of eggs that could be attributed to a single parent was 23 of the 30 assayed (for the parental genotype 139/153), so the nest's resident male could have fertilized at most about 77% of the eggs in the clutch (Table 4). Sneaking was not detected in the other nests collected without males (N1, N2, and N50; Table 3) because genotypes in the progeny arrays were consistent with a single father and mother.

There are two reasons why sneaking may have remained undetected in some of the attended nests as well. First, if a sneaker fertilized only a small fraction of the eggs, these might have remained unsampled. For example, a sneaker who fertilized only 1% of the eggs in a nest would have had (with probability 0.74) none of his offspring included in a random sample of 30 eggs from that nest. Second, a possibility exists that an embryo fathered by a sneaker male possessed a genotype compatible with its being an offspring of the resident male. To address this issue, we calculated two-locus exclusion probabilities (for *Fifsp10* and *Fifsp16*) for the 24 guardian males from which nests were analyzed (Table 2). Neither parent is known with certainty, so these exclusion probabilities differ from those reported in Table 1. The exclusion probability for a single locus is one minus the inclusion probability, which in this case is the proportion of unrelated embryos in the population that have either of the male's two

alleles at the locus in question. Under Hardy-Weinberg equilibrium, this inclusion probability is given by  $P(I) = (p_1 + p_2) [2 - (p_1 + p_2)]$ , where  $p_1$  and  $p_2$  are the frequencies in the embryo population of the male's two alleles. The multilocus inclusion probability is then the product of all single-locus inclusion probabilities, and the multilocus exclusion probability is one minus this value.

In our case, all of the two-locus exclusion probabilities calculated in this manner were 0.90 or greater (Table 2), with a mean of 0.956. Thus, if an unrelated embryo was sampled from a male's nest, we would expect to exclude it as a possible offspring of the nest-holding male in more than 95% of the cases. As more embryos are sampled from the sneaked nest, the probability of exclusion becomes increasingly similar to the probability of identity (Table 2) for the nest-holding male (i.e., the proportion of individuals in the population that share his multilocus genotype). This is because if a resident male and a sneaker both fertilized a large number of eggs from the same female that were exhaustively genotyped, the sneaker would have to share the same genotype as the resident male for the sneaking event to remain undetectable. Our conclusion from these statistical considerations is that although we may have failed to detect one or a few sneaking events involving a small proportion of sneaker-sired embryos, our estimate of an 18% sneaking rate for nests is probably close to the true value for this sample.

TABLE 4. Contents of the nests of males in which some progeny were deduced genetically to have resulted from sneaked fertilizations or from stolen egg clutches. Male IDs are as in Table 3. Shown are the numbers of clutches in each nest, eggs in each clutch, and for each clutch the number of embryos assayed and the proportion of these for which the resident male could not be excluded as the father. Two clutches in these nests were not developed sufficiently for microsatellite assay.

Male	(Clutch)	No. of eggs	No. embryos assayed	Proportion fertilized by nest-holder
<b>Sneaked</b>				
F1	(1)	724	30	0.57
	(2)	779	30	1.00
F8	(1)	746	30	0.70
	(2)	364	30	1.00
	(3)	643	30	1.00
F45	(1)	—	30	0.37
F51	(1)	411	27	0.63
	(2)	401	28	0.71
	(3)	287	30	1.00
N4	(1)	442	30	≤0.77
	(2)	264	0	—
<b>Stolen</b>				
F2	(1)	754	30	0.00
	(2)	128	30	0.00
	(3)	147	0	—
F37	(1)	117	30	0.00
	(2)	193	32	0.00
F46	(1)	475	31	0.00
	(2)	178	8	1.00
F66	(1)	582	30	0.00*

\* For two embryos in this nest, this resident male could not be excluded as a potential sire. Nonetheless, the most likely explanation for the observed progeny array is that all of the eggs were stolen (see text).

#### Egg Thievery by Males

We inferred thievery for a clutch of eggs when the guardian male was excluded as a father of nearly all of the embryos present (but see Discussion for other possible explanations). With our genetic data, the likelihood of overlooking this kind of event is low. Egg thievery would remain undetected only if, for each of the two assayed loci, either the father or the mother from which the clutch was stolen had a genotype identical to the stealing male. For two loci with numerous alleles and low identity probabilities, the likelihood of non-detection of egg thievery is about four times the probability of genotypic identity (Table 2). Thus, for our data, the likelihood of nondetection of a stolen clutch remains extremely low (*maximum* value about  $4 \times [1.4 \times 10^{-4}]$ ; Table 2).

Of the 24 nests with resident males, four (17%) contained stolen clutches. The issue of egg thievery cannot be addressed for the four nests collected without males because the genotype of the guarding male must be known to infer a stolen clutch. The nests of males F2, F37, and F66 apparently contained only stolen clutches (Table 4; note that one of the three clutches in F2's nest was not assayed), whereas the nest of male F46 contained one stolen clutch and one clutch that he had sired.

Male F66 could not be excluded as the father of two of the 30 embryos examined from his nest, despite the use of all six microsatellite loci in the assays. Inspection of the progeny genotypes revealed that for each embryo, either the

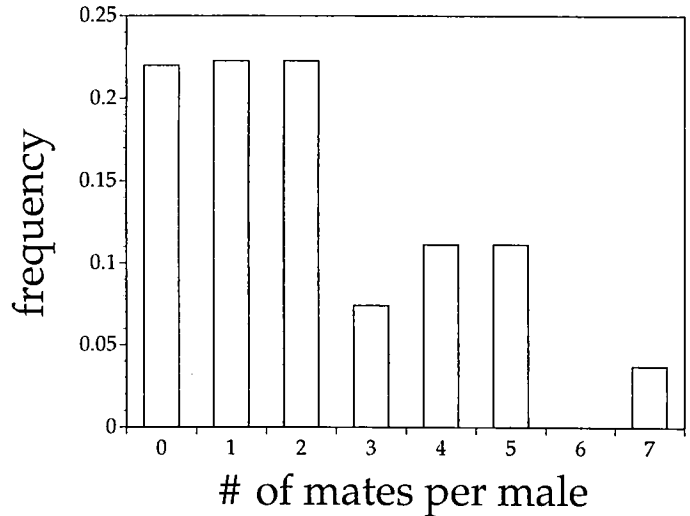


FIG. 2. Frequency histogram of male mating success (the number of females genetically inferred to have contributed to a male's brood) for assayed nests. Of course, males may have greater numbers of partners if observed throughout the entire breeding season. Males in the zero class either had no eggs or only stolen eggs in their nests.

inferred mother or the inferred true father shared at least one allele with F66 at each locus. Thus, we might expect some of the progeny not to be excluded even if F66 was not the true father. Based on these observations, this clutch probably was stolen but the possibility cannot be rejected that F66 sired some of the embryos assayed.

#### Male Reproductive Success

An added advantage of the highly variable markers employed in this study is that they permit estimates of the prevalence of multiple mating by males (Table 3). For nests in which the male was included as a father of some or all of the embryos, the maternal alleles present in the progeny array can be determined by subtraction. Furthermore, the association patterns of alleles at multiple loci within a nest allow reconstruction of multilocus genotypes for females that mated with the male in question (for methodological details, see Jones and Avise 1997b). Such analyses permit determination of the minimum number of females that contributed to each clutch.

Individual nests sampled in this study contained eggs from one to seven different females, with frequencies shown in Figure 2. These should be considered minimal estimates for two reasons. First, if two or more females with identical multilocus genotype contributed eggs to a nest, they would remain nondistinguished in the assays. However, given the low probabilities of genetic identity for our loci (Table 2), this complication should not be serious. Second, as the number of females who have mated with a male increases, our ability to detect the contributions of additional females decreases, both as a result of the increased probability that females share alleles with the male's other mates, and because clutches were not sampled exhaustively (see above).

No significant relationship was found between (1) male size and number of mates (regression;  $n = 22$ ,  $r^2 = 0.11$ ,  $P$

TABLE 5. Genotypic descriptions of inferred mates for five pairs of males who by genetic evidence in each case had mated with the same female. To be conservative, if only one maternal allele was observed in the progeny array, the female was assumed to have that allele and an unknown allele. The probability of identity is the probability that an individual of the given six-locus genotype would be drawn at random from the population. See Figure 3 for the spatial arrangement of these nests.

Male I.D. (clutch #)- female #	Female's genotype at microsatellite locus						Prob. of identity
	<i>Fifsp16</i>	<i>Fifsp10</i>	<i>Fifsp5</i>	<i>Fifsp1</i>	<i>Fifsp3</i>	<i>Fifsp15</i>	
F63 (1)-1	149/151	212/240	217/241	83/89	262/—	112/116	$1.12 \times 10^{-11}$
F62 (1)-1	149/151	212/240	217/241	83/89	262/—	112/116	
F48 (1)-1	147/157	226/248	223/243	89/—	262/266	114/—	$3.56 \times 10^{-9}$
F61 (1)-2	147/157	226/248	223/243	89/—	262/266	114/—	
F61 (1)-1	153/159	210/238	229/251	89/—	262/—	114/118	$9.86 \times 10^{-10}$
F62 (1)-2	153/159	210/238	229/251	83/89	262/—	114/118	
F46 (2)-1	153/157	192/258	229/237	83/89	262/—	112/116	$3.19 \times 10^{-10}$
F44 (2)-2	153/157	192/258	229/237	83/89	262/266	112/116	
F8 (1)-2	147/157	190/218	215/247	83/89	262/—	112/114	$2.73 \times 10^{-9}$
F2 (2)-1	147/157	190/218	215/247	83/89	262/—	112/114	

= 0.13); (2) male size and number of clutches in the nest ( $n = 23$ ,  $r^2 < 0.01$ ,  $P = 0.94$ ); and (3) male size and number of eggs in the nest ( $n = 17$ ,  $r^2 = 0.08$ ,  $P = 0.26$ ). There also was no significant difference in size among males with sneaked eggs (mean = 110.8 mm) versus stolen eggs (mean = 123.8 mm) versus those attending nests that were not involved in raiding (mean = 125.7 mm; ANOVA,  $P = 0.32$ ) or in the mean number of eggs in these three types of nests (ANOVA,  $P = 0.35$ ). All failures to detect significance might be due to the relatively small number of nests examined.

#### Female Mating Behavior

The hypervariable nature of the microsatellite loci also permitted inferences about the mating behavior of some females, despite the fact that no adult females were assayed directly in our analysis. As noted above, the genotypes of females that mated with assayed males could be inferred provisionally from the progeny arrays. A (reasonable) assumption underlying this inference is that males not excluded as sires of the progeny in their nests were indeed the true fathers of those embryos. By subtraction (and by the multilocus linkage disequilibrium observed among the maternal alleles within the progeny array), the genotypes of mothers then were reconstructed.

A total of 64 different mother genotypes were inferred in this study in initial assays based on the highly polymorphic loci *Fifsp10* and *Fifsp16*. In five cases, separate nests had mothers of the same inferred genotype, suggesting that the same female mated with both males. Genetic matches were verified by genotyping the four additional microsatellite loci in 10 embryos from each nest (Table 5). In each case, a perfect six-genotype match resulted. Because of the low expected frequencies of these genotypes in the population (between  $2.7 \times 10^{-9}$  and  $1.1 \times 10^{-11}$ ), it is nearly certain that specific individual females had deposited eggs in multiple nests. These results confirm that females of *S. spinachia* are capable of mating with multiple males during the span of a single embryo-incubation period. By reference to spatial maps of the nest collection sites (Fig. 3), two of the egg depositions were deduced to have involved short-distance movements (about 50 m), whereas the other two inferred movements involved distances of 500 and 1300 m.

#### A Sneaker Snared

Through a procedure analogous to that used to infer the genotypes of mothers, the genotypes of the sneaker males can be determined. From the resident male's genotype and those of his offspring, the female's genotype can be determined by subtraction, and then the sneaker male's genotype becomes evident. Of the four sneaker males for which the genotype could be determined (not possible for nest N4 because no resident male was captured), one sneaker (of F1) shared a two-locus genotype with one of the resident males in our collection (F55). The four additional loci then were assayed and a perfect six-locus match resulted. The expected population frequency of this genotype is  $1.1 \times 10^{-10}$ , again suggesting that it is probably unique in this local area. Thus, F55 was a territorial male who had stolen fertilizations in his neighbor's (F1's) nest, which was 7 m away from his own (Fig. 3).

#### DISCUSSION

The microsatellite genetic markers developed for *S. spinachia* proved extremely useful for analyses of nest-raiding behavior and mating success in nature. The frequency of nest raiding in this population was high, with 18% of nests containing progeny of sneaked fertilizations and an additional 17% containing stolen egg clutches. By assigning multilocus genotypes to the fathers and mothers of the progeny assayed, we were able to document multiple matings by females on a scale ranging from 50 m to more than 1 km, and we were able to identify one of the inferred sneakers as a resident of a nearby nest. The genetic analyses also documented that males of *S. spinachia* often mate with multiple females in a short time frame.

Previously little was known about the reproductive behavior of *S. spinachia*, and to our knowledge this study provides the first documentation of sneaked fertilizations and egg thievery by this species. Nest-raiding behaviors have been documented by direct observation in other stickleback species as well as in many other fish taxa. However, almost never have the genetic consequences of nest-raiding behavior been examined.

The fiftenspine stickleback may be similar in nest-raiding



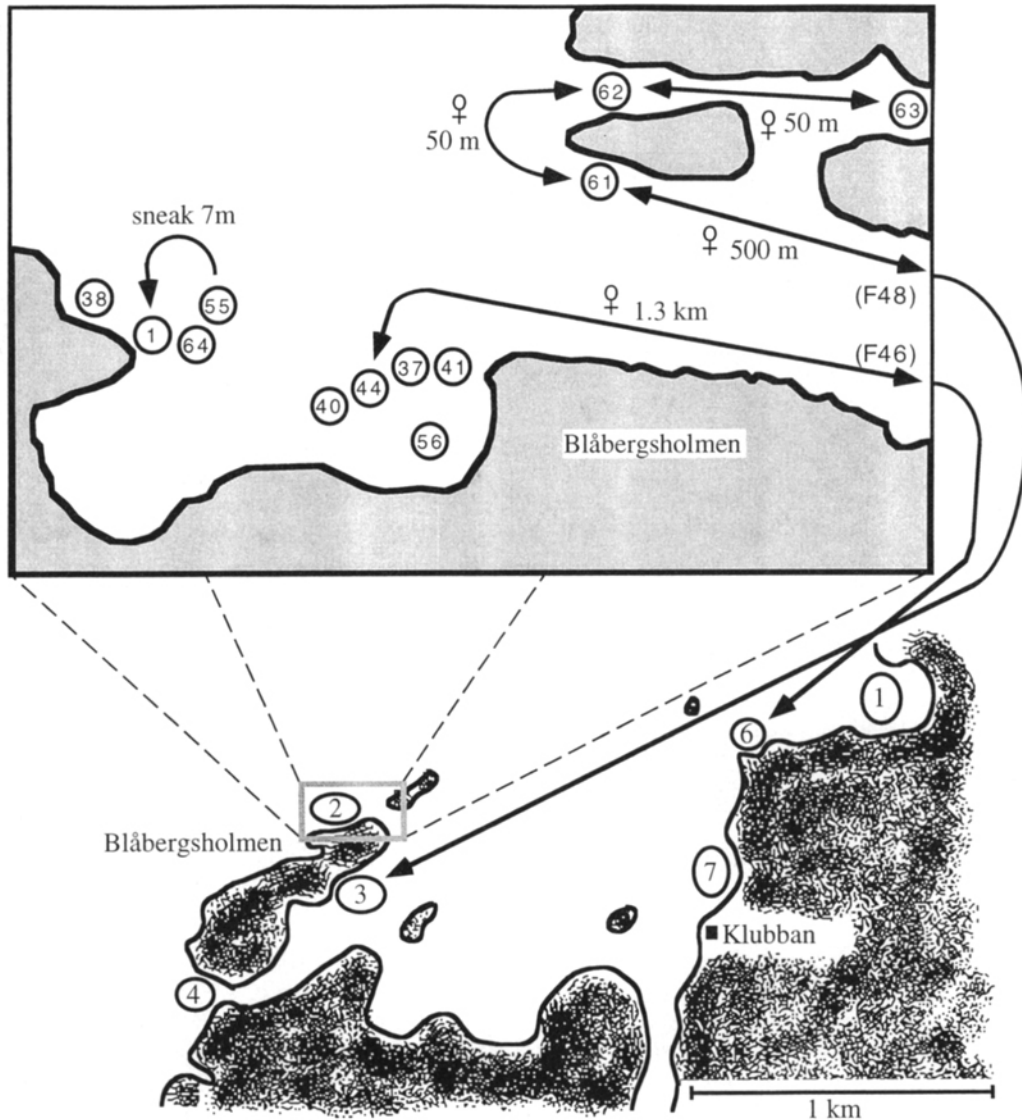


FIG. 3. Map of the collecting locale. Males were collected from seven different locations (numbered as in Table 3) outlined in bold on the coarse-focus map. Collecting locale 5 (not shown) was located 1.5 km off the lower left corner. The inset shows an enlargement of collection locale 2. Nests of the males (numbered as in Table 3) are shown as circles, with relative locations of nests drawn to scale. Double-headed arrows represent multiple matings by females who by genetic evidence laid eggs in more than one nest. Two arrows leaving the inset represent deduced movement by females who mated with males in different collecting locales. The single-headed arrow drawn from nest-holding male F55 to male F1 represents a genetically inferred event of sneaked fertilization by male F55.

behavior to the threespine stickleback, *G. aculeatus*, in which both sneaking and egg thievery also occur. In some populations of *G. aculeatus*, the frequency of sneaked nests can be as high as 35%, whereas in others the frequency is much lower (Goldschmidt et al. 1992). These figures are not directly comparable to our genetic findings because they are based on visual observations of sneakers whose genetic success rate remained unknown. However, Rico et al. (1992) used DNA fingerprinting to study sneaking and egg stealing in 17 nests of *G. aculeatus* (10 progeny assayed per nest). One nest contained eggs fertilized by a sneaker, two contained stolen eggs, and a fourth nest contained both stolen and sneaked eggs, yielding a total of 23% of nests that were raided. Our estimate of raided nests (about 35%) is somewhat

higher, but as our study did not address temporal or spatial variation in sneaking behavior, it remains possible that *S. spinachia* too exhibits much variation in nest-raiding activity.

Egg thievery also has been reported in other stickleback species, including the ten-spine (Morris 1952) and threespine (Whoriskey and FitzGerald 1994), but not in the four-spine (Willmot and Foster 1995). In threespine sticklebacks, egg thievery often accompanies sneaking behavior (Whoriskey and FitzGerald 1994): immediately after a sneaking male passes through the nest and sheds sperm, he may return to the entrance and remove eggs. If this happens with high frequency in *S. spinachia* as well, some uncertainty is introduced to interpretations in the current study. In nests containing embryos that by genetic evidence apparently were fertilized

by a sneaker, the resident guardian male himself actually may have been responsible for moving some of the "additional" eggs to his own nest.

In general, the fate of stolen eggs in sticklebacks is unknown, but laboratory experiments involving *G. aculeatus* have shown that the eggs usually vanish from the egg-stealer's nest or are eaten (Jamieson and Colgan 1992). However, in rare instances they may survive to hatch (Sargent and Gebler 1980). These issues related to egg stealing could be resolved by coupling additional laboratory or field observations of sneaked fertilizations and egg thievery with microsatellite assays to determine the parentage of the eggs involved.

We found a large number of apparently stolen eggs in our study, but several factors may have biased our estimate upward. We defined an egg-thievery event as one in which the resident male could be excluded as the father of an entire clutch. A highly efficient sneaking event in which the sneaker fertilized nearly all of the eggs would look like egg thievery by this definition. Although nothing is known about sperm precedence in sticklebacks, such an outcome might be possible because sneakers sometimes have been observed to pass through the nest and presumably release sperm prior to the resident male (Jamieson and Colgan 1992). Also, if a male adopted another's nest (as has been reported at low frequency in threespine sticklebacks; Mori 1995) and the nest already contained eggs from the original owner, the clutches would appear as if stolen in our analysis.

More conclusive evidence that a clutch was stolen would require a nest that contained two or more clutches of different age, and in which an elder clutch was sired by the resident male (from genetic evidence) and a younger clutch was fathered by a different male. This would suggest that the male had mated in the nest and was in its possession before the stolen clutch appeared. Of the four nests in our study with clutches suspected of being stolen, only one also contained an additional clutch that was fathered by the resident male. In this case, embryos in the suspected stolen clutch were more developed than those in the resident-fathered clutch. Nonetheless, given the high frequency of egg stealing observed in the laboratory and in nature in threespine sticklebacks and the fact that egg stealing has been observed in laboratory studies of this Swedish population of fifteen-spine sticklebacks (Östlund-Nilsson, unpubl. data), the clutches that appeared to be stolen in our sample most likely did, indeed, reflect egg thievery.

The reasons why these alternative reproductive behaviors exhibited by male sticklebacks should persist through evolutionary time are not immediately apparent. Phylogenetically, *S. spinachia* is thought to be the basal extant member of Gasterosteidae (McClennan 1993; Bowne 1994). By this evidence, and by the widespread distribution of nest-raiding behavior in sticklebacks, both sneaking and egg thievery appear to be ancestral conditions in the stickleback clade and presumably have persisted over great lengths of time. The theoretical benefit of sneaked fertilizations is obvious: a sneaker can increase his genetic fitness while simultaneously decreasing the reproductive success of a rival. The sneaker derives a further benefit in that he can avoid energetic investment in postzygotic care of his offspring. The strategies

of sneaking versus territoriality may constitute an evolutionarily stable strategy (ESS) in some species in which these reproductive modes are mutually exclusive (e.g., *Lepomis*: Gross 1982; *Salmo*: Hutchings and Myers 1988). However, previous observations of other stickleback species, together with our documentation of sneaking by a nest-holding male (F55), suggest that sneaking behavior in sticklebacks is a conditional, facultative strategy in which even nest-holding males can participate opportunistically (Foster 1994).

As a facultative strategy, sneaking should be maintained provided the benefit in some situations outweighs the cost. This condition is much easier to satisfy than those required for an evolutionary stable strategy (ESS), where hard-wired sneaker genotypes will be maintained only if the sneakers and territory holders have equal fitness or if there is frequency-dependent selection (Barnard and Sibly 1981; Gross 1991). For the fifteen-spine stickleback, it is instructive to consider the payoff in terms of fecundity to sneakers. In our sample, when sneaking was successful, the sneaker males fertilized on average 40% of the eggs in a clutch, suggesting that males benefit greatly by sneaking when an opportunity presents itself. However, only 5% (66 of 1307) of the total eggs assayed in this study were the result of sneaked fertilizations, suggesting that sneaking cannot serve many males in the population as a primary reproductive strategy. To interpret these numbers further, more must be learned about the costs associated with sneaking behavior, the frequency of attempted sneaking in the field, and the conditions under which males attempt to sneak fertilizations.

The evolutionary basis of egg stealing is even more puzzling. Why would a male steal and perhaps care for eggs that were fertilized by a rival? In many fish species, females prefer to mate with males whose attended nests already contain eggs (Bisazza and Marconato 1988; Sikkell 1989). Another possible advantage of egg stealing involves predation dilution. By adding unrelated eggs to his nest, a male may decrease the vulnerability of his own eggs to predation (Whoriskey and FitzGerald 1994). Most baffling, however, is why a male might steal eggs that he potentially fertilized through sneaking behavior (see above), when he otherwise could leave them to the care of another male.

The facultative sneaking behavior of the fifteen-spine stickleback is not unlike sneaking behavior and EPCs of many taxa. Egg stealing, however, is a rarer phenomenon. Perhaps one reason that egg thievery occurs in sticklebacks and not in other taxa is that sticklebacks have portable egg masses. In many taxa, for example, egg thievery simply is impossible owing to eggs being large and cumbersome or small and loose. The genetic consequences of egg thievery resemble those of intraspecific brood parasitism (IBP) in birds (Petrie and Møller 1991; McRae and Burke 1996) or adoption in mammals (McNutt 1996), that is, the resident caregiver is not the biological parent of the progeny receiving the care.

In birds, IBP has a potential for relatively clear fitness advantages for the egg-dumping female. This raises an interesting question for species such as sticklebacks in which external clutches of eggs are highly portable: Do males sometimes move eggs from their own nests into those of other males? Although our data cannot rule out this possibility, this

kind of parasitism has not been reported by behavioral biologists working with sticklebacks.

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