The genetic mating system of spotted sunfish (*Lepomis punctatus*): mate numbers and the influence of male reproductive parasites

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Abstract

In nest-building fish species, mature males often exhibit one of two alternative reproductive behaviours. Bourgeois males build nests, court females, and guard their eggs. Parasitic cuckolders attempt to steal fertilizations from bourgeois males and do not invest in parental care. Previous evidence from the bluegill sunfish (*Lepomis macrochirus*) suggests that adult males are morphologically specialized for these two tactics. Here, we used microsatellite markers to determine genetic parentage in a natural population of the spotted sunfish (*L. punctatus*) that also displayed both bourgeois and parasitic male morphs. As gauged by relative investments in gonadal vs. somatic tissues, between 5 and 15% of the mature adult males were parasites. Multi-locus genotypes were generated for more than 1400 embryos in 30 nests, their nest-guardian males, and for other adults in the population. Progeny in approximately 57% of the nests were sired exclusively by the guardian male, but the remaining nests contained embryos resulting from cuckoldry as well. Overall, the frequency of offspring resulting from stolen fertilizations was only 1.3%, indicating that the great majority of paternity is by bourgeois nesting males. With regard to maternity, 87% of the nests had at least three dams, and computer simulations estimate that about 7.2 dams spawned per nest.

Keywords: cuckoldry, evolutionarily stable strategy (ESS), maternity, mating tactics, microsatellite, paternity

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Introduction

Organisms have evolved varied mating strategies in response to selection pressures promoting reproductive success. Differences between the sexes with regard to reproductive behaviours have long been of interest (Darwin 1871; Fisher 1958), but recently, increased attention has been devoted to mating tactics within a gender (Gibbs *et al.* 1990; Gross 1996; Henson & Warner 1997; Mauck *et al.* 1999). In particular, the evolution and maintenance of alternative reproductive strategies within a sex has received considerable attention (see for example, Zimmerer & Kallman 1989; Gross 1991; Lucas & Howard 1995).

One such classic example of alternative mating strategies among males involves the bluegill sunfish *Lepomis macrochirus* (Gross 1979; Dominey 1980). For males of reproductive age, individuals can be classified as either bourgeois or parasitic based on behaviour, appearance, and relative investments in gonadal vs. somatic tissues (Gross & Charnov 1980; Taborsky 1997). Bourgeois males build nests, court females, vigorously defend eggs and juveniles from intruders, and have a low ratio of testes to body size. Parasitic males steal fertilizations from bourgeois males via female mimicry or by simply streaking through the nest and releasing gametes while the bourgeois male is actively spawning with a female (Dominey 1980; Gross 1982; Ehlinger *et al.* 1997). In some populations, as many as 85% of the reproductively active males display the parasitic lifestyle (Gross 1982).
In theory, the relative success of the bourgeois strategy may be related to the frequency of parasitic cuckolders in the population (Gross 1991; Philipp & Gross 1994), if for no other reason than that paternity by bourgeois males is high when sneaker morphs are absent (Philipp & Gross 1994; DeWoody et al. 1998). Recently, we used microsatellite markers to document parentage in a related species, the redbreast sunfish (L. auritus). Roughly 40% of the nests surveyed showed evidence of cuckoldry. However, based on morphology and colouration, no male reproductive parasites were observed in the study population. We suspected that reproductive parasitism in L. auritus probably stems from bourgeois males themselves.

Here, we document the existence of alternative male reproductive strategies (bourgeois and parasite) in a population of the spotted sunfish (L. punctatus). We suspected that the presence of a cuckold morph in the spotted sunfish might result in even higher incidences of cuckoldry than those found in the redbreast sunfish. We generate and employ microsatellite data to document genetic parentage for large numbers of embryos within numerous spotted sunfish nests. These data permit estimates of cuckoldry rates and the number of females who contributed to each nest. They also prompt comparisons of these findings to those for the redbreast sunfish likewise assayed from the same stream in South Carolina (DeWoody et al. 1998).

Materials and methods

Field collections and laboratory techniques

Samples were collected in May and June 1998 from three different sites (1, 2, and 3) in Fourmile Creek, a tributary of the Savannah River (Fletcher et al. 2000). Guardian males were captured on 29 nests, using techniques described in DeWoody et al. (1998). Also collected (for identification of parasitic males and allele frequency estimates) were a total of 83 adult females and 44 reproductively mature males that were collected indiscriminately from known spawning areas. Embryos/larvae scooped from the nest substrate were preserved in an ambient solution of 20% DMSO/saturated NaCl.

The reproductive phenotypes of field-collected adult males were categorized as either ‘bourgeois’ or ‘parasitic’ based on the gonadosomatic index (GSI; Jennings & Gross 1992a; Taborsky 1998), which is the weight of an individual’s gonadal tissue expressed as a percentage of total body weight. Often, the GSI for reproductive parasites is at least two times higher than those of bourgeois males (see Gross 1982; Taborsky 1998). Thus, the GSI tends to be strongly bimodal and nonoverlapping (Ehlinger et al. 1997), thereby providing a clear demarcation between individuals specialized for the two alternative reproductive tactics.

DNA was extracted by standard organic, Chelex, or embryo extraction protocols (Manitatis et al. 1982; Altschmied et al. 1997; DeWoody et al. 2000a). Embryos were genotyped at two microsatellite loci (R87 and R820) originally developed for Lepomis auritus (DeWoody et al. 1998), but in this case the primers were labelled with fluorescent dyes. Reactions were electrophoresed in 4.5% denaturing polyacrylamide gels on an ABI 377 automated sequencer, and alleles were sized with respect to electrophoretic mobility. Genotypes were assigned using the Genotyper (ABI) software package.

Genetic assessments of parentage

Utilizing the allele frequencies observed in the adult population, computer simulations were employed to estimate the mean sample sizes necessary to detect all maternal contributions to the progeny array within a nest (DeWoody et al. 2000b). These simulations suggested that if no more than nine females contributed equally to a nest, a sample of 49 embryos per nest would be sufficient on average to detect the gametic contributions of each dam. If fewer than nine females normally contributed to a nest, smaller samples of offspring should suffice. Based on the results of these a priori simulations, we genotyped a mean of 48 embryos per nest.

Genotypes were scored for each embryo and its nest-attendant male. Typically, most of the progeny within a given nest proved to be either full- or half-sibs, so maternal contributions to each embryo were readily deduced by subtraction. The minimum number of dams contributing to a nest was tallied by taking the smallest integer value greater than or equal to one-half the number of different maternal genotypes represented in the progeny array (e.g. DeWoody et al. 1998; Kellogg et al. 1998). Di-locus matrix procedures (conceptually similar to Punnett squares) assisted in reconstructing the relative contributions of different mothers to each nest (DeWoody et al. 2000c). To adjust the minimum number of dams toward more realistic estimates of the true number of mothers per nest, the computer simulations of DeWoody et al. (2000b) were employed.

Results

Population characterization

Among 44 males (collected from all three sites) not affiliated with the assayed nests, 42 (95%) were classified by morphology as bourgeois, and only two (5%) as potential reproductive parasites (Fig. 1). Both of these parasites were found at site 1 in Fourmile Creek, yielding

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an estimate of 15% for the frequency of parasitic sneaker phenotypes at this specific locale. Because no parasitic sneaker morphs were found at the other two sites (2 and 3), overall at least 85% of the reproductively mature, nonterritorial males collected from all three sites in Fourmile Creek appeared to be of the bourgeois ilk. Two more parasitic cuckolders were also collected during the course of broader fish collections, and GSIs for these fish are included in Fig. 1 for illustrative purposes. However, because those two parasites were captured during non-targeted sampling of fish from the stream, they are not included in the current frequency estimates. Nonetheless, these general collections support the notion that parasitic males are rare compared to bourgeois males.

The four individuals displaying the parasitic morphology were small [48, 51, 51, and 55 mm in standard length (SL)], barely half the length of the nest-guarding males collected (Table 1). They also were smaller than most mature females in the population (mean SL = 70; SD = 14, range = 46–118). These parasites did not express any of the sexually dimorphic characters (copper breast colouration, black pelvic fins, or enlarged ear tabs) typical of bourgeois males. Body dimensions of the adult reproductive parasites were similar to those of juvenile fish of similar size. Many juveniles, however, were larger than the parasites. Thus, the parasitic morph is not part of an obligate ontogenetic state (sensu Jennings & Philipp 1992a) as occurs in some minnows (Fletcher & Burr 1992). Presumably, as juveniles destined to become bourgeois males mature, they develop with age and size a deeper body characteristic of the bourgeois morph.

The two microsatellite loci (RB7 and RB20) yielded 19 and 16 alleles, respectively, in our sample of 39 adults collected near nests (Fig. 2). Assuming that one parent (i.e. the father) is known with certainty, the average single-parent exclusion probability (Selvin 1980) for the two loci exceeded 0.95. Alternatively, when both parents are unknown, the average parentage exclusion probability (Garber & Morris 1983) is 0.88. In practice, these numbers may underestimatethe exclusionary power of the genetic assays because not all alleles present in the sunfish population were represented in our sample of adults; in many progeny arrays, we detected maternally derived alleles not present in the adult population sample. Thus, a more exhaustive population survey would likely reveal even more allelic diversity which could further increase the exclusion probabilities.

**Paternity**

All of the surveyed progeny in 17 of the 30 nests (57%) displayed multilocus genotypes that were consonant with paternity by a single male, usually the guardian (Table 1). [In nest #19, no guardian male was collected, but all juveniles had genotypes consistent with a single sire.] Thirteen of these 17 nests (76%) were from site 1. In a statistical comparison between sites at which parasitic morphs either were or were not found, the frequencies of cuckolded nests were not demonstrably different (binomial, \( P > 0.1 \)).

Low levels of cuckoldry were detected in the 13 other nests (43%) surveyed (Table 1). For example, two of 47 embryos (4.2%) surveyed from nest #12 clearly were sired by a male other than the nest attendant (Fig. 3). Overall, of the 13 cuckolded nests, nine (69%) were from site 1. In a statistical comparison between sites at which parasitic morphs either were or were not found, the frequencies of cuckolded nests were not demonstrably different (binomial, \( P > 0.1 \)).
Despite the fact that roughly 43% of the nests contained juveniles which were genetically inconsistent with the guardian male, paternity by the guardian male was generally very high (Table 1). For example, the most severe case of nonpaternity by any single resident bourgeois male occurred in nest #23, where only three of 44 embryos (7%) were inconsistent with that attendant’s genotype.

In one of the cuckolded nests (#15), none of the embryos was sired by the attendant male. However, 46 of those 48 embryos (96%) were consistent with paternity by a single other hypothetical male. Of the two remaining embryos from that nest, one was provisionally excluded as an offspring of the primary sire on the basis of a single locus, and the other was excluded at both loci. Thus, one male (not the captured guardian) likely sired 46 of the sampled offspring and a second male (a cuckolder of the primary sire) fathered the other two embryos assayed. Hence, the primary sire was expelled, departed, or died, and nest #15 then was taken over by the captured guardian.

Table 1

| Nest no. | Site no. | Standard length | Paternity | Minimum no. of dams | Estimated no. of dams |
|----------|----------|----------------|-----------|---------------------|----------------------|
| LP-1     | 1        | 104            | 57 of 58† | 6                   | 10.8                 |
| LP-2     | 1        | 109            | 50 of 51† | 6                   | 11.8                 |
| LP-3     | 1        | 103            | 59 of 59  | 5                   | 9.7                  |
| LP-4     | 1        | 131            | 55 of 55  | 5                   | 8.0                  |
| LP-5     | 1        | 113            | 25 of 25† | 3                   | 3.3                  |
| LP-6     | 1        | 101            | 37 of 37  | 4                   | 4.8                  |
| LP-7     | 1        | 103            | 47 of 47  | 6                   | 12.7                 |
| LP-8     | 1        | 97             | 35 of 35  | 4                   | 5.4                  |
| LP-9     | 1        | 84             | 46 of 46  | 1                   | 1.0                  |
| LP-10    | 1        | 90             | 55 of 56† | 5                   | 8.0                  |
| LP-11    | 1        | 95             | 54 of 54  | 6                   | 11.5                 |
| LP-12    | 1        | 92             | 45 of 47† | 5                   | 11.0                 |
| LP-13    | 1        | 100            | 52 of 54† | 5                   | 10.7                 |
| LP-14    | 1        | 87             | 88 of 89† | 4                   | 5.8                  |
| LP-15    | 2        | 93             | 46 of 48†§ | 4                  | 4.8                  |
| LP-16    | 2        | 91             | 47 of 47  | 2                   | 2.1                  |
| LP-17    | 1        | 97             | 47 of 48† | 5                   | 9.2                  |
| LP-18    | 1        | 98             | 41 of 42† | 6                   | 10.3                 |
| LP-19    | 1        | no guardian    | 45 of 45  | 4                   | 4.3                  |

*Nest not sired by attendant male, but most of the progeny are consistent with paternity by a single male (suggesting a nest takeover event; see text).
†Embryos excluded on the basis of locus RB20 only; mutation possible as an alternative explanation.
‡Entire nest surveyed.
§Embryos excluded at both loci, cuckoldry.
¶Embryos excluded on the basis of locus RB7 only; mutation possible as an alternative explanation.

Fig. 2 Allele frequencies for microsatellite loci RB7 and RB20 in 39 presumably unrelated Lepomis punctatus adults from Fourmile Creek.
de novo mutations could account for some of the paternity exclusions. However, using these same microsatellite loci, DeWoody et al. (1998) argued (on the basis of known patterns of microsatellite mutations) that single-locus exclusions are more likely due to cuckoldry. Thus, in the current context, we assume that all exclusions are due to stolen fertilizations rather than mutations. Either way, the fact remains that bourgeois nesting males generally sire more than 98% of the juveniles in the population.

**Maternity**

In each of two nests (#9 and #25), the progeny array contained no more than two maternal alleles at a locus, or four di-locus gametotypes. Thus, these nests appear to have a single mother and father. Furthermore, the full-sib progeny in each case were sired by the guardian male. Within these nests (which perhaps were incomplete with respect to spawning), all embryos were of the same developmental phase. Nests #9 and #25 both contained substantially fewer embryos (probably less than 300 individuals) than did most other nests (>500 embryos), except nest #30 (see Table 1).

Two other nests were likely mothered by just two females each. Nest #16 consisted of embryos from two different dams, one of whom contributed approximately 69% of the eggs (the other, 31%). Similarly, two dams contributed to nest #20 in a ratio of 75:25. Table 2 illustrates the reconstruction of maternal genotypes and estimation of reproductive skew in nest #16.

In contrast, all other nests (26 of 30, or 87%) contained more than four maternally derived gametes and, thus, contained eggs from at least three dams (Fig. 4). Whereas estimates for the minimum number of mothers are unambiguous, the reconstruction of maternal genotypes (and determining the true number of mothers) is often not...
straightforward (Table 3). The ambiguity results from the fact that as more parents contribute to a progeny array, the probability that two or more parents share alleles increases dramatically (Fiumera et al. 2001). Thus, we used computer simulations to estimate the true number of dams for each nest (DeWoody et al. 2000b,c). Results are summarized in Fig. 4. In some cases, the estimated number of dams was almost twice the minimum number (Table 1).

Discussion

Theory suggests that the ‘optimal’ reproductive strategy of any organism depends largely on the frequency and success of all competing strategies in the population (Gross 1991), which themselves may be partial functions of ecological circumstance. In some species (such as Lepomis macrochirus), alternative male reproductive strategies are thought to be both genetic and obligatory (Dominey 1980; Gross 1982). In such cases, it follows that if parasitic males can achieve reproductive success only by stealing fertilizations from bourgeois males, then forces promoting

Table 2 Maternal gametotypes and female reproductive skew in nest LP16. One female (with genotype 125/129 at locus RB7, 236/272 at RB20) contributed 69% of the embryos surveyed from the nest, and the other (di-locus genotype of 113/121, 248/284) contributed the remaining 31%. Gametic phase disequilibria were not significant

| Locus (RB7) | 125 | 129 | 113 | 121 |
|-------------|-----|-----|-----|-----|
| 236         | 7   | 6   | 3   | 1   |
| 272         | 5   | 9   | 4   | 2   |
| 248         |     |     |     |     |
| 284         |     |     |     |     |

Fig. 4 Distributions of the minimum (above) and estimated (below) number of mothers per nest across the 30 surveyed nests of Lepomis punctatus.

Locus RB20

| Locus (RB20) | 280 | 330 | 248 | 256 | 240 | 288 | 284 | 264 | 244 | 260 | 300 |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 121          | 5   | 4   |     |     |     |     |     |     |     |     |     |
| 129          | 3   | 4   |     |     |     |     |     |     |     |     |     |
| 145          | 5   | 2   |     |     |     |     |     |     |     |     |     |
| 193          | 3   | 2   |     |     |     |     |     |     |     |     |     |
| 173          | 5   | 2   |     |     |     |     |     |     |     |     |     |
| 181          |     |     |     |     |     |     |     |     |     |     | 1   |
| 117          |     |     |     |     |     |     |     |     | 1   |     |     |
| 133          |     |     |     |     |     |     |     |     |     |     |     |
| 149          |     |     |     |     |     |     |     |     | 3   |     |     |

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GENETIC PARENTAGE AND CUCKOLDRY IN SUNFISH

Male reproductive behaviour

In our study population of *L. punctatus*, 13 of 30 nests showed evidence for reproductive parasitism, yet more than 98% of the 1434 embryos surveyed were sired by the bourgeois, nest-attendant males. In other words, despite the moderate incidence of cuckoldry (43% of the nests), less than 2% of the progeny could have stemmed from reproductive parasitism by sneaker (or other) males.

Theory predicts that stable genetic equilibria between competing reproductive strategies can occur when (in this case) the lifetime fitness of parasitic sneakers and bourgeois males are equal to the proportions of eggs they each fertilize (Gross 1991; Berglund 1997). In our study sites at Fourmile Creek, between 5 and 15% of the reproductively mature males in our samples were of the parasitic morph. The estimates are only modestly larger than the suspected frequency of progeny (1.3%) that might have been conceived by parasitic sneakers. Thus, with current data, we cannot entirely eliminate the possibility that the coexistence of bourgeois and parasitic males in our population of *L. punctatus* is an example of a mixed evolutionarily stable strategy (ESS; Maynard Smith 1982) governed by a genetic polymorphism for parasitic vs. bourgeois lifestyles. A similar suggestion has been made by Gross (1991) for *L. macrochirus*.

Alternatively, the sneaker vs. bourgeois morphs in *L. punctatus* might result from environmental influences to which embryos, fry, or young adults are exposed, perhaps triggering ontogenetic switches. If so, any genetic underpinning for the sneaker/bourgeois dichotomy might be reflected as a scope for developmental plasticity rather than a hardwired genetic polymorphism (Ehlinger et al. 1997). Perhaps such possibilities could be tested empirically by rearing fry under controlled laboratory conditions, as has been done to document developmental plasticity for distinct trophic morphs in some cichlid fishes (see Sage & Selander 1975; Turner & Grosse 1980; Meyer 1987).

Female reproductive behaviour

Compared to male sunfish, less is understood about the reproductive ecology of females. It is known that female sunfish often distribute their eggs among several nests (Gross 1991), and that a female may spawn with the same male in temporally discrete reproductive bouts (DeWoody et al. 1998). However, if a large number of females typically spawn in a nest, reconstruction of precise maternal genotypes within a progeny array is difficult even with highly polymorphic microsatellite markers, and it is seldom possible to be certain of the exact number and contributions of dams for each nest (Table 3; DeWoody et al. 2000a,c).

Nonetheless, it is clearly the case in our study population that multiple females often contributed eggs to a nest (Fig. 3). Indeed, only two of 30 nests contained embryos from a single female. The difference between the minimum and the true number of females to spawn in a nest depends upon the variability of the markers employed to infer maternity (DeWoody et al. 2000b), and the two estimates may differ substantially (Fiùmera et al. 2001; Table 1). In the present study, however, as many as six different females demonstrably spawned in particular nests, and the computer simulations suggest that the true number sometimes may exceed a dozen females per nest. This notion is reinforced by the fact that we based our sampling regime on the assumption that fewer than 10 females spawned (on average) in each nest. Had we accounted for more females in our initial simulations (and thus sampled more progeny per nest), estimates of the number of females spawning in each nest might be even larger (DeWoody et al. 2000b).

In many fish species, females are attracted to larger males, such that one potential predictor of both male and female reproductive success is body size (Jennings & Philipp 1992b). In the present study, the standard lengths of nest-tending males were regressed against the number of females spawning in each nest might be number sometimes may exceed a dozen females per nest. This notion is reinforced by the fact that we based our sampling regime on the assumption that fewer than 10 females spawned (on average) in each nest. Had we accounted for more females in our initial simulations (and thus sampled more progeny per nest), estimates of the number of females spawning in each nest might be even larger (DeWoody et al. 2000b).

Interspecific comparisons and environmental factors

Parentage patterns in the spotted sunfish from Fourmile Creek are strikingly similar to those of redbreast sunfish sampled from the same drainage. Thus, based on directly comparable genetic assays and analysis procedures for the two species, the documented (minimum) mean number of dams per nest was 3.7 in the redbreast sunfish, and 4.4 in the spotted sunfish. Likewise, the incidences of cuckoldry (proportions of nests with at least some progeny not sired by the attendant male) were 40% and 43%, and the overall percentages of offspring resulting from cuckoldry were roughly 10% and 2% in the two respective species. Nest takeovers (Taborsky 1994) also were observed in both species.

One apparent difference between the redbreast and spotted sunfish is that morphs specialized for the male
reproductive parasitic tactic are not reported in *L. auritus*. For the bluegill sunfish, Philipp & Gross (1994) suggested that reproductive parasitism is directly proportional to sneaker densities. However, the similarities between the redbreast and spotted sunfish with regard to paternity in Fourmile Creek suggest that cuckoldry rates are influenced by factors in addition to sneaker density.

For *L. auritus*, nonpaternity by attendant males appears to increase as a function of nest density (as opposed to sneaker density), suggesting that the former is a key component of cuckoldry success (Fletcher *et al.* 2000). This hypothesis is supported by the fact that guardian males steal fertilizations from nearby nesting males when opportunities arise (Jennings & Philipp 1992b; DeWoody *et al.* 1998; Taborsky 1998). Why do males build colonies if their paternity assurance decreases with increasing nest density? Of course, high nest densities are a double-edged sword for males, so one possibility is that certain males also may benefit from colonial nesting by virtue of their added scope for fatherhood via cuckoldry of neighbours.

Another set of possibilities relates to potential advantages for females. Coloniality may benefit females because of reduced predation (perhaps a dilution effect, or joint defence), less male sexual harassment, and/or better opportunities for mate choice (Berglund 1997; Danchin & Wagner 1997). In some fish and bird species, females (rather than males) choose mating sites and female activity can determine which nest sites are desirable (Warner 1987; McKaye *et al.* 1990; Warner 1990; Stauffer *et al.* 1995; Wagner *et al.* 2000). Thus, colonialism may be a male response to intersexual selection pressure operating via female choice (Danchin & Wagner 1997; Henson & Warner 1997).

In *L. auritus*, nests in colonial settings contain, on average, progeny from more females than do solitary nests (DE Fletcher, unpublished data). This fact may contribute to male tolerance for colonial spawning in the face of increased cuckoldry pressure. However, the results from generally solitary nesting *L. punctatus* (Carr 1946; present study) suggest that the nearest-neighbour distance had little effect on the number of mates (data not shown) and that most nest-building males in Fourmile Creek, regardless of proximity to other bourgeois males, regularly spawn with at least half a dozen females.

Conclusions

In summary, as gauged by morphological evidence, spotted sunfish in Fourmile Creek display two distinct male mating strategies (bourgeois and parasitic). The genetic data demonstrate that a great preponderance of paternity is by the bourgeois (nest-attendant) strategy, and that most nests are mothered by multiple (>5) dams. These conclusions about genetic parentage are remarkably similar to those for redbreast sunfish studied at the same site. Further research is required to determine if the bourgeois/parasitic polymorphism in the spotted sunfish is genetically "hard-wired" or, alternatively, reflects a developmental or behavioural switch.

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