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Genetic monogamy and biparental care in an externally fertilizing fish, the largemouth bass (*Micropterus salmoides*)

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Breeding, male North American sunfish (Centrarchidae), are often brightly coloured and promiscuous. However, the largemouth bass (*Micropterus salmoides*) is sexually monomorphic in appearance and socially monogamous. Unlike some other nest-tending centrarchids in the genus *Lepomis*, largemouth bass have also been reported to provide biparental care to eggs and fry. Here we use microsatellite markers in order to test whether social monogamy predicts genetic monogamy in the largemouth bass. Offspring were collected from 26 nests each usually guarded by a pair of adults, many of which were also captured. Twenty-three of these progeny cohorts (88%) proved to be composed almost exclusively of full-sibs and were thus the product of monogamous matings. Cuckoldry by males was rare. The genetic data also revealed that some nests contain juveniles that were not the progeny of the guardian female, a finding that can be thought of as low-level ‘female cuckoldry’. Overall, however, the data provide what may be the first genetic documentation of near-monogamy and biparental care in a vertebrate with external fertilization.

**Keywords:** maternity; paternity; cuckoldry; mating system; sexual selection

1. INTRODUCTION

Modes of fish reproduction are highly diverse (Breder & Rosen 1966; Baylis 1981; Helfman et al. 1997). Fertilization may be internal or external, mating may be monogamous or promiscuous and there may be biparental care of offspring, uniparental care by either sex or no parental care at all. This diversity provides a rich conceptual arena for examining sexual selection and the evolution of mating systems.

Sexual selection theory suggests that monogamy typically constrains fitness differences among males whereas polygamous mating systems can magnify such variation (Darwin 1871; Bateman 1948; Mock & Fujikawa 1990). The evolution of social monogamy among fishes, although relatively rare, has been evidenced by detailed ecological data on several model species (Barlow 1992; Wiegmann et al. 1992; Reavis & Barlow 1998; Annett et al. 1999). However, there is great potential in externally fertilizing fishes for extra-pair spawning and intraspecific brood parasitism. In particular, nesting males are vulnerable to cuckoldry and molecular studies on other organismal groups (e.g. birds and mammals) have shown that observed mating behaviours do not always directly translate into corresponding patterns of genetic parentage (Gowaty & Karlin 1984; Colman et al. 1999).

Only a few studies have assessed genetic mating systems in fishes and most of these have dealt with polygamous species (Parker & Kornfield 1996; DeWoody et al. 1998, 2000; Kellogg et al. 1998). Jones et al. (1998) documented genetic monogamy in a seahorse species with internal fertilization, but there have been no such molecular assays of externally fertilizing vertebrates that are thought to be monogamous.

One socially monogamous fish with external fertilization is the largemouth bass, *Micropterus salmoides*. Each breeding male occupies a territory and tends a nest. Unlike the bowl-shaped depressions scooped out by male *Lepomis* sunfish, bass nests are crude and may be constructed on logs or large rocks (Reighard 1906; Breder & Rosen 1966). A successful male fertilizes eggs when a gravid female releases her clutch during the courtship ritual (Breder 1936). Ecological evidence suggests that *Micropterus* males are only rarely bigamous (Ridgway et al. 1989; Wiegmann et al. 1992). Interestingly, there are historical and anecdotal reports of biparental care of young by largemouth bass (Smith 1907; Hankinson 1908; J. R. Baylis, personal communication). Overall, bass are probably the most attentive parents in the sunfish family, often remaining with schooling fry for as long as one month after hatching (Reighard 1906; Breder 1936; Wiegmann & Baylis 1993).

Here, we used molecular markers in order to critically evaluate the mating system of *M. salmoides*. We collected offspring and guardian adults from nests in nature and then used microsatellite analyses to determine parentage and reproductive success. The genetic mating system is discussed in light of sexual selection theory.

2. MATERIAL AND METHODS

(a) Field collections

We collected nest-attendant adults and their custodial offspring from the Steel Creek drainage, a tributary of the...
Savannah River on the US Department of Energy’s Savannah River Site near Aiken, South Carolina, in April and May 1998 (when water temperatures ranged from 19°–23°C). Offspring were taken from 23 nests in the spillway below the L-Lake dam in shallow water (mean ± s.d. = 82 ± 19 cm) near the bank (2.2 ± 0.9 m). Six nests were also taken from L-Lake proper in shallow water (61 ± 19 cm) significantly further from shore (6.3 ± 1.4 cm). Nests averaged 67 ± 30 cm in diameter and were found on solid objects such as boulders, logs and stumps (n = 7), in sand and gravel near the base of solid objects (n = 13) or among fine roots at the base of emergent macrophytes (n = 8). Nests contained anywhere from ten to several thousand offspring. One of the nests actually consisted of a free-swimming school of fry that were 10–20 mm in length. A guardian male tenaciously defended these fry from conspecifics as well as from \textit{Lepomis} adults that were actively attempting to eat them. Another large bass stayed consistently within \textit{ca}. 1 m of the school. This fish was probably a female and the guardian male made no attempt to chase her. After the attendant male was caught, the other guardian became much more aggressive in defence of the fry, chasing away bluegill and other bass. We collected much of the school but unfortunately were unable to catch this key individual.

Adults were captured from nests by electrofishing (DeWoody \textit{et al}. 1999) or hook and line. Midway through our collecting efforts, we noticed that females as well as males apparently guarded nests. Generally, they were observed facing the nest from 1–2 m distance, whereas attendant males maintained a position directly over the nest (although occasionally this pattern was reversed). Guardian males did not try to chase away the nest-guarding female (as they did other conspecifics). Guardian females were aggressive towards conspecific intruders as well as embryo-eating predators such as \textit{Lepomis}, suggesting that they were actively tending juveniles and not simply mate guarding. For example, in the nest consisting of free-swimming fry, when the guardian male was caught, the other guardian (presumably a female) became much more aggressive in defence of the fry. The general behaviour of these female bass closely resembled that previously described for males in a nest-tending cichlid fish, \textit{Tilapia mariae} (Annett \textit{et al}. 1999).

### Laboratory techniques

Embryos and/or larvae scooped from the nest substrate were preserved in an ambient solution of 20% dimethyl sulphoxide/saturated NaCl. DNA was extracted by the methods cited in DeWoody \textit{et al}. (2000) or by boiling dissected embryos for 20 min in a solution of 5% chelex (w/v with water). Five dinucleotide microsatellite loci (table 1) were cloned from a largemouth bass library via conventional procedures (e.g. Choudhary \textit{et al}. 1993) and flanking polymerase chain reaction (PCR) primers were designed. Most of the bass specimens were genotyped using four of these loci (table 1), which incidently also amplified DNA from the bluegill \textit{Lepomis macrochirus}. In addition, one tetranucleotide locus (RB7) cloned from redbreast sunfish (\textit{Lepomis auritus}) (DeWoody \textit{et al}. 1998) was employed in the current assays of bass. Loci MS21 and MS25 were amplified with Promega’s \textit{Taq} DNA polymerase (1 unit per reaction), Promega 10X buffer and 2.0 mM MgCl$_2$. The other three loci were amplified using 1 unit of \textit{Taq} per reaction and LGL 10X buffer (0.5 M KCl, 0.1 M Tris 8.0, 15 mM MgCl$_2$ and 0.5 mg ml$^{-1}$ BSA). Loci MS19 and RB7 were multiplexed using a twofold molar excess of the RB7 primers; MS13 and MS25 were only multiplexed if the embryos were sufficiently developed for yielding large quantities of template DNA. Fluorescent dye-labelled PCR products (0.3–0.5 μl) were electrophoresed on an ABI 377 sequencer and genotypes were scored using the manufacturer’s software.

### Genetic assessments of parentage

If the genotypes of embryos in a nest were consistent with the multilocus genotype of the guardian male, he was provisionally deemed the genetic sire. Likewise, in cases where an attendant female was captured and her multilocus genotype was consistent with the progeny array, she was considered the genetic dam.

#### Table 1. Microsatellite primers for largemouth bass

(The values for the PCR profiles are the times (s) spent denaturing at 94°C/annealing at a designated temperature/extension at 72°C. $P_E$ is the probability of exclusion when one parent is known (from equation (1) in DeWoody \textit{et al}. (2001)). The combined probability of exclusion was 0.92. Locus MS23 was not optimized, but preliminary results suggested that it may be polymorphic.)

<table>
<thead>
<tr>
<th>locus/primers</th>
<th>sequence (5'→3')</th>
<th>fluorescent label</th>
<th>PCR profile</th>
<th>number of alleles</th>
<th>$P_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB7F</td>
<td>GTGCTAATAAAGGCTACTGTC</td>
<td>—</td>
<td>30/30/30 at 47°C for 27 cycles</td>
<td>10</td>
<td>0.40</td>
</tr>
<tr>
<td>RB7R</td>
<td>TGTTCCTTAATTGGTTTGA</td>
<td>6-FAM</td>
<td>—</td>
<td>5</td>
<td>0.38</td>
</tr>
<tr>
<td>MS13F</td>
<td>CTGATACAGCAGCTGGAAGC</td>
<td>NED</td>
<td>30/30/30 at 53°C for 30 cycles</td>
<td>11</td>
<td>0.64</td>
</tr>
<tr>
<td>MS13R</td>
<td>CTTCTGCTCC/GCATCGCTCTTAG</td>
<td>—</td>
<td>30/30/30 at 47°C for 27 cycles</td>
<td>8</td>
<td>0.35</td>
</tr>
<tr>
<td>MS19F</td>
<td>CAGGATTTGAACTAGCCAGGC</td>
<td>HEX</td>
<td>30/30/30 at 54°C for 30 cycles</td>
<td>11</td>
<td>0.64</td>
</tr>
<tr>
<td>MS19R</td>
<td>GGAATGATGATGATGTTGTTGTA</td>
<td>HEX</td>
<td>30/30/30 at 50°C for 27 cycles</td>
<td>6</td>
<td>0.35</td>
</tr>
<tr>
<td>MS21F</td>
<td>CACCTGAATGGCAGCTGTTG</td>
<td>6-FAM</td>
<td>2/2/8 at 58°C for 25 cycles</td>
<td>11</td>
<td>0.64</td>
</tr>
<tr>
<td>MS21R</td>
<td>GTTGTGACGTCAGTCTGCCG</td>
<td>—</td>
<td>—</td>
<td>2</td>
<td>0.08</td>
</tr>
<tr>
<td>MS23F</td>
<td>CATAGGATGTCGCGTGAAC</td>
<td>6-FAM</td>
<td>30/30/30 at 50°C for 27 cycles</td>
<td>11</td>
<td>0.64</td>
</tr>
<tr>
<td>MS23R</td>
<td>GATTACTTACACAGGTCC</td>
<td>—</td>
<td>30/30/30 at 50°C for 27 cycles</td>
<td>11</td>
<td>0.64</td>
</tr>
</tbody>
</table>

the attendant male proved to be the sire but no putative mother was identified (or vice versa), the maternal (or paternal) contributions to each embryo were simply deduced by subtraction.

At least one of the parents was captured at each nest almost without exception and the other could be genetically deduced from the collective genotypes of the progeny array. However, if neither parent was captured, the Mendelian assortment of independent loci made it impossible to reconstruct the joint multilocus paternal and maternal genotypes that produced a full-sib cohort (although the shared parent’s genotype can usually be inferred from half-sib progeny cohorts) (figure 1). Nevertheless, such progeny arrays were considered to be composed of full-sibs if no more than four alleles were present at any one locus and if alleles were distributed among juveniles in a manner consistent with Mendelian inheritance from two parents.

3. RESULTS

Multilocus genetic data were gathered for a total of 1088 juvenile largemouth bass representing 25 nests and the one school of fry. Twenty-five guardian males associated with these progeny arrays and six attendant females that we managed to capture were also genotyped.

(a) Population characterization

Five of the loci proved to be polymorphic in this largemouth bass population, with three to 11 alleles each (table 1). All loci appeared to assort independently and no null alleles were in evidence. The exclusion probabilities (Garber & Morris 1983; DeWoody et al. 2001) ranged from 0.08 to 0.64 and the combined probability across five loci was 0.92 (table 1). The standing levels of microsatellite variation in our population of *M. salmoides* were typical of most freshwater fishes (DeWoody & Avise 2000).

(b) Genetic paternity

Twenty-one out of the 25 primary attendant males assayed appeared to have sired all of the progeny sampled within their respective nests (table 2). These genetic
findings are fairly straightforward, so we need only elaborate on the four nests (nests 1, 2, 6, and 104) in which genetic paternity by the guardian male was in doubt.

On first inspection, nests 1 and 2 did not appear to be sired by their guardians (table 2). However, each of these nests was composed exclusively of full-sib embryos. Furthermore, the genotype of the guardian male of nest 1 was entirely consistent with the genotypes of all progeny in nest 2 and, conversely, the genotype of the guardian of nest 2 was consistent with the genotypes of all progeny in nest 1. The four-locus identity probabilities for these two guardian’s genotypes were $8.5 \times 10^{-4}$ and $3.6 \times 10^{-4}$, respectively. Thus, these two males or their nests were probably inadvertently mislabelled (switched) at some point. If so, then, in 23 out of the 25 progeny arrays (92%) for which a guardian male was captured, those nest-attendant males apparently sired all of the embryos within their respective nests.

Thus, both full-sibs and half-sibs were present within the nest, suggesting that one parent was cuckolded (see §4(b)).
The situation in nest 104 was more complicated (table 2). From genetic evidence, this nest contained embryos from two different mothers. The guardian male apparently fertilized all five of the embryos sampled from one of these females, but only 33 of 48 embryos from the other. A likely interpretation is that the 15 embryos for which the guardian male was genetically excluded were the result of stolen fertilizations (cuckoldry) by another male.

On 6 May, no male was collected (or observed) on nest 15, which nonetheless contained exclusively full-sib embryos. However, a female attendant (who proved to be the mother) was captured from that nest and this allowed the paternal genotype to be deduced from the progeny array. Interestingly, this deduced paternal genotype (probability of identity = 3.8 × 10⁻³) (table 3) was identical to that observed in the male guardian of nest 18, who was collected on 8 May. Nest 18 was first observed on 7 May, suggesting that a single male spawned in nest 15 and subsequently respawned in nest 18 after our collections disrupted its original nest.

Altogether, if one disregards nest 6 (and also nest 15 for which a guardian male was not captured), then the 24 nest-tending bass males assayed in this study proved to have sired 968 out of the 983 embryos (98.5%) sampled from their respective nests. Only a few remaining embryos (ca. 1.5%) from a single nest (104) were the probable result of male cuckoldry.

(c) Genetic maternity

Several insights about maternity in this bass population likewise came from the genetic analyses, either from genotypes observed directly in nest-tending females or from mothers’ genotypes, as deduced from progeny arrays for which the sire was known.

Six reproductively mature females were captured as putative guardians in association with particular nests. Three of these were proved to be the genetic mothers of all embryos sampled from their respective nests by genetic evidence, but in the other three cases this was not invariably true (table 2). In nest 6, as already mentioned, the attendant female was not the mother of any embryos in the nest, suggesting that the true mother had departed or been displaced. Likewise in nest 21, the putative guardian female collected was not the mother of any of the embryos assayed. Finally, the female guarding nest 18 was genetically excluded as the true mother of embryos sampled from that nest, but her multilocus genotype clearly pointed to her as the deduced mother of nest 10 (table 3). Nest 10 was sampled on 4 May and no guardian female was collected. Three days later, we collected nest 18 and its guardian female, which turned out to be the mother of the embryos found in nest 10. Thus, our collection efforts appear to have driven the female from nest 10 to nest 18, where she was apparently ready to respawn.

Interestingly, the deduced (i.e. unsampled) mother of nest 18 was genetically excluded as the true mother for only one of the 38 embryos sampled from nest 18. Similarly, in nest 24, which was actually a school of fry, the deduced genotype of the mother of 39 full-sib juveniles clearly excluded her as the mother of one other sampled progeny. These incidences may reflect cases of ‘female cuckoldry’, wherein a second female may have laid a few eggs into the nest of the guardian male who sired all of the progeny.

We collected an attendant female without a guardian male in only one nest (nest 15). The genetic data indicated that this guardian female was indeed the mother of these embryos and that she had spawned in that nest with a single male (who, as mentioned before, had apparently sired all embryos sampled in nest 18 as well).

(d) Spawning in multiple nests

In total, we deduced or directly obtained multilocus genotypes for 55 reproductively mature bass. Only five pairs of genotypes matched one another in 1485 pairwise comparisons between these (table 3). Four of these matches indicated that individual bass spawned in more than one nest and the fifth match was between the deduced mother of nest 10 and the female guardian of nest 18 (who was not the genetic mother of those embryos).

The multilocus genotypes of deduced mothers or guardian females matched one another in three instances (table 3), indicating that individual females occasionally spawned in more than one nest. One such instance of a genetic match involved the captured attendant female of nest 11, who apparently spawned with the guardian male of that nest in addition to the male guardian of nest 3. In two other such cases, an unsampled female appears to have spawned in both nests 4 and 20 and another unsampled female spawned in nests 9 and 24. As indicated earlier, these repawnings may have been unintentionally induced by our collection efforts.

Only one male appeared to spawn in more than one nest. The guardian male captured on nest 18 not only sired the embryos from that nest, but also all of the embryos sampled from nest 15 (table 3).

4. DISCUSSION

According to the genetic evidence, the nest-tending largemouth bass in our study population had an
unusually high assurance (by externally fertilizing fish standards) of biological parentage for the embryos they guarded (DeWoody & Avise 2001). Furthermore, individuals were seldom genetically documented to have spawned in multiple nests. Thus, males typically spawned with only a single female and females with only a single male (table 2). That these outcomes are unusual in fish species surveyed to date is underscored by the contrast with confamilial sunfish in the genus *Lepomis*. There, from comparable kinds of genetic evidence, particular males often spawn with more than half a dozen females, each of whom may spawn with several males (DeWoody & Avise 2001). Furthermore, in the current study, our collecting efforts themselves were probably responsible for some (if not all) of the rare instances in which an individual largemouth bass respawned in another nest.

A net effect of bass monogamy was that the collections of progeny within 23 out of the 26 nests (88%) were composed entirely or almost entirely of full-sibs. In only four nests were progeny other than full-sibs observed in appreciable frequency and three of those cases resulted when an attendant male had clearly spawned with two or three females, leading to the presence of some half-sib nest-mates. These findings support behavioural observations indicating that largemouth bass are socially (and perhaps serially) monogamous and they document the genetic consequences of this behaviour.

(a) Monogamy, biparental care and sexual monomorphism

Barlow (1986) noted that monogamous fish tend to share several features, including large body size, seasonal breeding, possession of breeding territories, demersal eggs and biparental care extending beyond the egg stage to the protection of free-swimming fry. Our data (both ecological and genetic) on largemouth bass, coupled with previous field observations of biparental care in this species (Smith 1907; Hankinson 1908; J. R. Baylis, personal communication), are entirely consistent with Barlow’s (1986) view. Strong ecological evidence for monogamy has also been presented in a related species, the smallmouth bass (*Micropterus dolomieui*) (Wiegmann et al. 1992; Wiegmann & Baylis 1995), although nest defence in that species is thought to be uniparental.

Monogamy can be driven by mate guarding (Brotherton & Rhodes 1996), but our behavioural observations and genetic data indicated that both male and female largemouth bass often provide parental care to their own embryos. Biparental care and monogamy are relatively rare among fishes (Blumer 1979; Baylis 1981; Barlow 1986) and the two have long been thought to be codependent. However, recent evidence suggests that monogamy can also exist with uniparental care (Wiegmann et al. 1992; Wiegmann & Baylis 1995; Brotherton & Rhodes 1996; Jones et al. 1998).

Sexual dimorphism often evolves in response to sexual selection via male–male competition or female choice. In fishes, sexual selection often leads to the evolution of bright colours in males or other body adornments such as showy fins (Darwin 1871; Breder & Rosen 1966). *Micropterus* adults are sexually monomorphic and monogamous, whereas many other centrarchids (e.g. *Lepomis*) are distinctly sexually dimorphic and polygynous (DeWoody & Avise 2001). Analogous relationships between sexual dimorphism and genetically deduced degrees of polyandry across species also have been noted in the sex role-reversed syngnathid fishes (Barlow 1992; Jones & Avise 2001).

(b) Female cuckoldry

Two bass nests (18 and 24) contained single offspring that were genetically excluded (in each case at three microsatellite loci) as progeny of the primary female, indicating that a second mother contributed to these nests. The embryos excluded may simply have resulted from multiple mating by the male but, if so, the female reproductive skew in each nest approaches 40:1 (table 2). Two other nests (6 and 104) likewise contained small fractions of embryos derived from an additional mother.

This pattern of low-level multiple maternity and strong maternal reproductive skew in each of several nests is quite analogous to the genetic signature of male cuckoldry in *Lepomis* sunfish (DeWoody et al. 1998, 2000). In externally fertilizing species such as the largemouth bass, the coexistence of monogamy and biparental care raises the intriguing possibility that a ‘cuckolder’ female, by laying some eggs in a foreign nest, may in effect steal fertilizations and capture some parental investment by same-sex competitors, thereby reaping reproductive benefits similar to those of cuckolder males that are common in many other fish species. There may be many behavioural nuances to such female cuckoldry. For example, larger (and more fecund) nestling female bass may be the preferred mates of guardian males, in which case smaller females may attempt to cuckold large spawning females. More extensive molecular assays coupled with field observations should be able to determine whether this or other such possibilities are true.

5. SUMMARY

We provide, what is to our knowledge, the first genetic documentation of near-monogamy in an externally fertilizing animal. The evidence for largemouth bass consists of nest collections usually composed entirely of unique suites of full-sib embryos. The argument for monogamy is supported by our field observations of parental care, where females as well as males often tended what proved to be their genetic young. Fertilization thievery by males was rare (but did occur in one nest) and the genetic data are consistent with infrequent cuckoldry by females as well.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.