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Spawning Behavior in a Non-Teleost Actinopterygian: Genetic Evidence for Both Monogamy and Polygamy in *Amia*

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The bowfins, *Amia*, have been regarded as regarded as promiscuous or polygynous spawners, although this has not been directly observed. Species of *Amia* spawn largely at night in male-constructed and male-guarded nests, making it difficult to view spawning behavior. Furthermore, in many species, field observations of spawning behavior do not always match genetic observations. To investigate the mating system of *Amia*, we collected fin tissue from guarding males and a sample of associated eggs or fry from 15 broods to assess the number of parents involved in each brood. Using these tissues, we genotyped individuals at 11 microsatellite DNA loci. The genotype of the male was known; we inferred the genotype of the primary female by subtraction, using the most abundant fry genotypes and the known male genotype. We inferred polygamy (presence of multiple fathers or multiple mothers) in cases where there was more than a single fry at a single locus for which the genotype could not be explained by primary parental genotypes. Among sampled broods, seven contained offspring of a single male–female pair. Fry from eight broods show evidence of polygamy: in one brood, there was evidence of an extra-pair male; in five broods, there was evidence of at least one additional female, and in two of these five broods, there was also involvement by at least one additional male; in two broods, it could not be determined whether the additional parent was male or female. Thus, nearly half of our sampled broods were both behaviorally and genetically monogamous, while other broods were genetically polygamous. It has been suggested previously that female bowfin may spawn with more than one male, but there are no behavioral observations to support this claim and our genetic data cannot discern whether females are spawning in more than one nest. We conclude that *Amia* is behaviorally monogamous and weakly genetically polygynous. With respect to the evolution of mating systems of fishes, it is interesting that *Amia*, representing a member of the Holostei or the sister group to teleosts, is behaviorally monogamous, albeit with opportunistic participation in spawning by either extra males or extra females or both. A review of the literature on spawning behavior of non-teleostean ray-finned fishes suggests that additional behavioral and genetic studies of these fascinating taxa are warranted.

AMIA, commonly known as bowfins, are large charismatic freshwater fishes native to eastern North America. *Amia* currently includes two extant species, *Amia calva* and the recently resurrected species, *Amia ocellicauda* (Brownstein et al., 2022; Wright et al., 2022). Voracious predators, bowfins have been regarded by some as "undesirable" species (Dean, 1898; Stacy et al., 1970) or "rough fish" (Rypel et al., 2021). However, their value as sport fishes (Adams and Hankinson, 1928; Cermele, 2017; David et al., 2018) as well as their ecological roles in maintaining relative abundance among other species have also been recognized (Becker, 1983; Scarnecchia, 1992). As food fishes, some have described bowfins as unpalatable (Dean, 1898) while others describe them as tasty when properly prepared (Coker, 1918; Smith, 1994), and, more recently, bowfin caviar has been marketed successfully (Fritchey, 2006). Thus, there is increased interest in bowfins in the context of fisheries and aquatic ecology.

Although many aspects of the natural history and anatomy of bowfins have been well studied (e.g., Dean, 1898; Reighard, 1903; Jarvik, 1980; Ballard, 1986; Grande and Bemis, 1998), there is renewed interest in their biology (Dornburg et al., 2021; Thompson et al., 2021; Brownstein et al., 2022; Mikami et al., 2022; Wright et al., 2022) due to "the light the biology of this key species sheds on the evolution of ray-finned and bony vertebrates in general" (Thompson et al., 2021). Gars and bowfins occupy a pivotal phylogenetic position as members of the Holostei, the closest living taxon to the entire clade of teleost fishes (Normark et al., 1991; Grande, 2010; Near et al., 2012; Hughes et al., 2018). Furthermore, bowfins and gars have held interest as "living fossils" representing actinopterygian (ray-finned) fishes outside the teleostean clade (Dean, 1898; Schultze and Wiley, 1984; Dornburg et al., 2021; Wright et al., 2022; but see Clarke et al., 2016). As a member of the sister group to teleosts and one of only a handful of extant non-teleostean actinopterygians (i.e., bichirs, sturgeons, paddlefishes, gars, and bowfins), all aspects of bowfin biology and natural history, including reproductive traits such as parental care and mating system, provide important comparative context for understanding the evolution of those same traits among teleosts.

Within teleost fishes, there exists a considerable diversity of mating systems (Breder and Rosen, 1966; Turner, 1986; Helfman et al., 2009), and an increasing number of these

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have been characterized or refined through genetic analysis (for reviews, see DeWoody and Avise, 2001; Avise et al., 2002; Coleman and Jones, 2011; Rueger et al., 2019; Francis et al., 2022). One important finding from studies of the mating systems of teleosts and other animals is that genetic data do not always agree with observational data (DeWoody and Avise, 2001; Avise et al., 2002; Farias et al., 2015; Rueger et al., 2019). Thus, genetic analyses of mating systems is of interest among the ray-finned fishes outside the teleosts, as well as within teleosts, and an eventual understanding of which traits persist phylogenetically or arise convergently requires study of every relevant taxon. Here, we use genetic data to illuminate the bowfin mating system, which previously had only been the object of conjecture.

Bowfins are nesting species exhibiting male parental care (Dean, 1898; Reighard, 1903; Breder and Rosen, 1966). However, little is known about their mating system because they most often spawn at night, making direct observations of spawning rare. Females are infrequently observed at nests because they are transient, visiting to spawn and then departing. In Reighard's (1903, p. 80–81) classic and detailed study of 177 nests, he directly observed only four cases of spawning, all of which involved a single male–female pair (Reighard, 1903). In modern terms, this simple male–female spawning would be considered monogamy, under the definition of "heterosexual pairs exclusively mating with one another for at least one breeding cycle" (Wilson, 1975; Rueger et al., 2019).

Despite only observing spawning by single pairs, Reighard speculated that more than one female may spawn with one male and a single female might spawn in the nests of several males. He based this conjecture on the fact that nest-guarding sunfish males had been observed to mate with multiple females (polygyny), that there were large numbers of eggs in some bowfin nests, and that eggs were developing synchronously in adjacent nests. Obviously, inferring the mating habits of one species from another distantly related and ecologically distinct species would not be convincing now. However, subsequent authors conveyed Reighard's speculation as fact, with Breder and Rosen (1966) writing that a bowfin "male may spawn with several females and a female may spawn in several nests." This was not inconsistent with an early observation of three fish (sex not identified) on a nest during spawning, causing Dean (1898, p. 250) to suggest that bowfins spawn in "spawning parties," although Breder and Rosen (1966) dismissed this observation as an "abnormality." Subsequently, secondary sources have regarded *Amia* as polygynous, promiscuous, or polygamous (e.g., Gill, 1905; Smith, 1985; Jenkins and Burkhead, 1994; Burr and Bennett, 2014). To our knowledge, no one has specifically suggested that bowfins are monogamous or polyandrous and, despite Reighard's careful monographic study, current characterization of their mating system as polygynous, polygamous, or promiscuous has not yet been confirmed by either behavioral or genetic observations.

In the decade we have been visiting Oneida Lake, New York to search for nests of bowfins, now considered *A. ocellicauda* (Wright et al., 2022), to collect eggs for evolutionary developmental work (Cass et al., 2013; Funk et al., 2020a, 2020b, 2021), we have never observed spawning and we have rarely seen a female closely associated with a nest. Even so, the accessibility of males guarding eggs and fry on nests constructed in shallow water provided an opportunity to investigate mating in *Amia* with genetic data. To assess whether *Amia* is monogamous or polygamous and in what way, we captured males and a sample of their associated offspring and then assayed their genotypes. For each male-brood pair, we determined genetically whether there were more than two parents represented in one brood. We employed two different types of genetic markers: allele calls at diploid microsatellite loci and size variation in the (presumably maternally inherited) mitochondrial D-loop region. These two modes of inheritance enable us to distinguish monogamy from polygamy and in some cases to distinguish polyandry from polygyny for bowfin nests exhibiting polygamous mating.

MATERIALS AND METHODS

***Study system: background on the reproductive ecology of* Amia.**—Burr and Bennett (2014) provide a recent comprehensive species account of *Amia calva*, relying substantially on Reighard's (1903) extensive monograph, which remains an important primary source for the reproductive ecology of *Amia*. A summary of Reighard's pertinent observations, slightly updated, as noted by our experience, follows. During the spring (late April and early May in upstate New York), reproductively active bowfin males are easily distinguished from females by their bright green paired fins, green belly, and intensely colored ocellus on the caudal peduncle (Fig. 1A). Males tend to be smaller than females. Males construct concave, approximately round or multi-lobed nests in shallow marshy areas, tributary streams, or the vegetated edges of lakes. Nests are constructed in water depths as little as 15–20 cm. Nests are often located in places that afford some shelter under overhanging vegetation, fallen tree trunks, and at the bases of stumps, banks, or logs, but they can also be in relatively open areas close to shore. Nest building occurs primarily at night (Reighard, 1903, p. 72). Males remove most vegetation from the nest bottom, and exposed roots are often stripped to white (Fig. 1B). Occasionally, nests seem not be to be constructed; rather the males make use of existing natural depressions (Reighard, 1903, p. 74). Males will guard nests prior to spawning but appear to be more tenacious about guarding the nest after spawning when they are guarding eggs or fry (A. McCune, pers. obs.; Fig. 1C). Females are apparently transient, departing after they spawn. Gill (1905, p. 432) distilled Reighard's description of courtship and spawning thus: "after a period of longer or shorter play caressing and circling about, the two come together side by side, the one laying the eggs, the other fertilizing" and lasting as long as 80 minutes, or perhaps overnight. After the initial pairing, the same male and female may spawn repeatedly, up to five or six times (Reighard, 1903, p. 79–80; confirmed by Ballard, 1986, p. 338). Reighard (1903, p. 67) reported the greatest spawning activity with water temperatures between 16–19°C, and the earliest nests seen with water temperatures at 12°C, all temperatures taken at 1800 h. We found males guarding eggs in temperatures as low as 9–12°C, with temperatures taken between 1000–1100 h (McCune, unpubl. data). As described by Reighard (1903), newly spawned eggs stick to substrate or vegetation and are creamy white (Fig. 2A), turning to a much less visible gray within a day (Fig. 2B). After hatching, larvae lie motionless in the substrate and are nearly translucent save for the yolk sac, then darkening as pigment cells develop (Reighard, 1903; A. McCune, pers. obs.; Fig. 2C).

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Fig. 1. Roving and guarding male bowfin in spawning coloration. (A) Male swimming in Big Bay Creek, New York. Note bright green fins and the prominent ocellus on the caudal peduncle, both characteristic of spawning coloration (photo copyright David O. Brown, using scuba). (B) Male lying on constructed nest with stripped roots (photo by A. McCune using an Olympus Stylus Tough 8000 camera). (C) Male guarding fry on his nest. Black fry are seen in contrast to the brown substrate and are in a round patch underneath the male from approximately the snout to just anterior to the pelvic fins (photo by A. McCune).

We found these newly hatched larvae to be very cryptic. On the nest, the male guards the eggs, and hatched fry (about 7 mm long), for about eight to ten days (Reighard, 1903). Wriggling and actively swimming fry are jet black and highly visible (Fig. 1C). After eight to ten days, the male will venture from the nest with his ball or traveling school of black fry (Fig. 2D), which, by that stage, are about 11–12 mm or larger (Reighard, 1903). Males continue to care for fry until they are as much as 100 mm long (Reighard, 1903; Forbes and Richardson, 1908).

Sample collection.—According to recent work, the species of *Amia* in Oneida Lake, New York we studied is properly referred to as *A. ocellicauda* (Brownstein et al., 2022; Wright et al., 2022). We genotyped guarding males and a sample of their fry (or in one case, eggs) from nests of *A. ocellicauda* in Big Bay Creek, at the northwest end of Oneida Lake, New York. We also genotyped a lake-wide sample of adults, both male and female. Fin clips were used as genetic material in all adult samples. Overall, fin clips were taken from 93 adults collected between 28 April 2009 and 18 May 2012. Seventy-five of these were captured at varied geographic locations in the lake. This "lake-wide" sample of 75 adults was made during two time periods: between 18 September 2009 and 13 October 2009 (12 males and 6 females) and between 11 October 2010 and 23 October 2010 (21 males and 36 females). Lake-wide sampling represented equal effort in eight sections around the lake to ensure that sampling was unbiased relative to nearness to the spawning grounds at the northwest section of the lake. These 75 adults were captured primarily by electrofishing, although two adult females were caught in a trap net off Shackelton Point on the south shore on 23 October 2010. In addition to the "lake-wide" sample, we collected 18 guarding males, along with eggs or fry from their nests. While we attempted to collect males guarding a nest as well as fry he was guarding in all cases, males sometimes eluded capture, and we were not able to find eggs or fry in every guarded nest, presumably because the eggs or fry were cryptic gray, or transparent, or spawning had not yet occurred. Adult males were captured by angling and/or hand netting while males were guarding nests between late April and late May over three years (2009, 2011, 2012). Approximately 20–30 fry (or in one case, eggs) from the nests of 13 of the 18 nest-guarding males were genotyped for 11 microsatellite loci. A sample of 20 fry was genotyped from a free-swimming fry ball and associated male collected on 30 May 2011; the adult male in this case was not captured. A sample of 30 fry was sampled from nest 13 in 2012 but the guarding male escaped (Table 1). During field work, nests were numbered and recorded along with the year the nest was found (e.g., 2012–13 was the 13th nest found in 2012).

A piece of caudal fin approximately 10 mm × 5 mm was taken from each adult and stored in individually numbered vials containing 95% ethanol for genomic DNA extraction. Samples of offspring were either plucked from the nest substrate or netted and stored in 95% ethanol. Twenty offspring from each group genotyped were measured for total length. Fin and offspring samples were stored for up to two years at 4°C prior to DNA extraction. Offspring and fin clip tissues have been vouchered at the Cornell University Museum of Vertebrates (CUMV) as listed in Table 1. Institutional abbreviations follow Sabaj (2020). Field notes have also been provided to the CUMV.

PCR amplification and sequencing of mitochondrial cytochrome oxidase I and D-loop sequences.—A fragment of the mitochondrial cytochrome oxidase I (COI) gene was PCR-amplified from a lake-wide subset of 24 adults. PCR primers were designed with PrimerSelect software from complete mitochondrial DNA sequences of *Amia calva* published at the National Center for Biotechnology Information (NCBI) website. The forward primer sequence was 5′–ACAAAGACATTGGCACCCTCTACCTA–3′ and the reverse primer sequence was 5–GTTTTGCTGCGAAAGCTTCTCATAGTA–3′. Ten microliter PCR reactions consisted of 1x Platinum Taq

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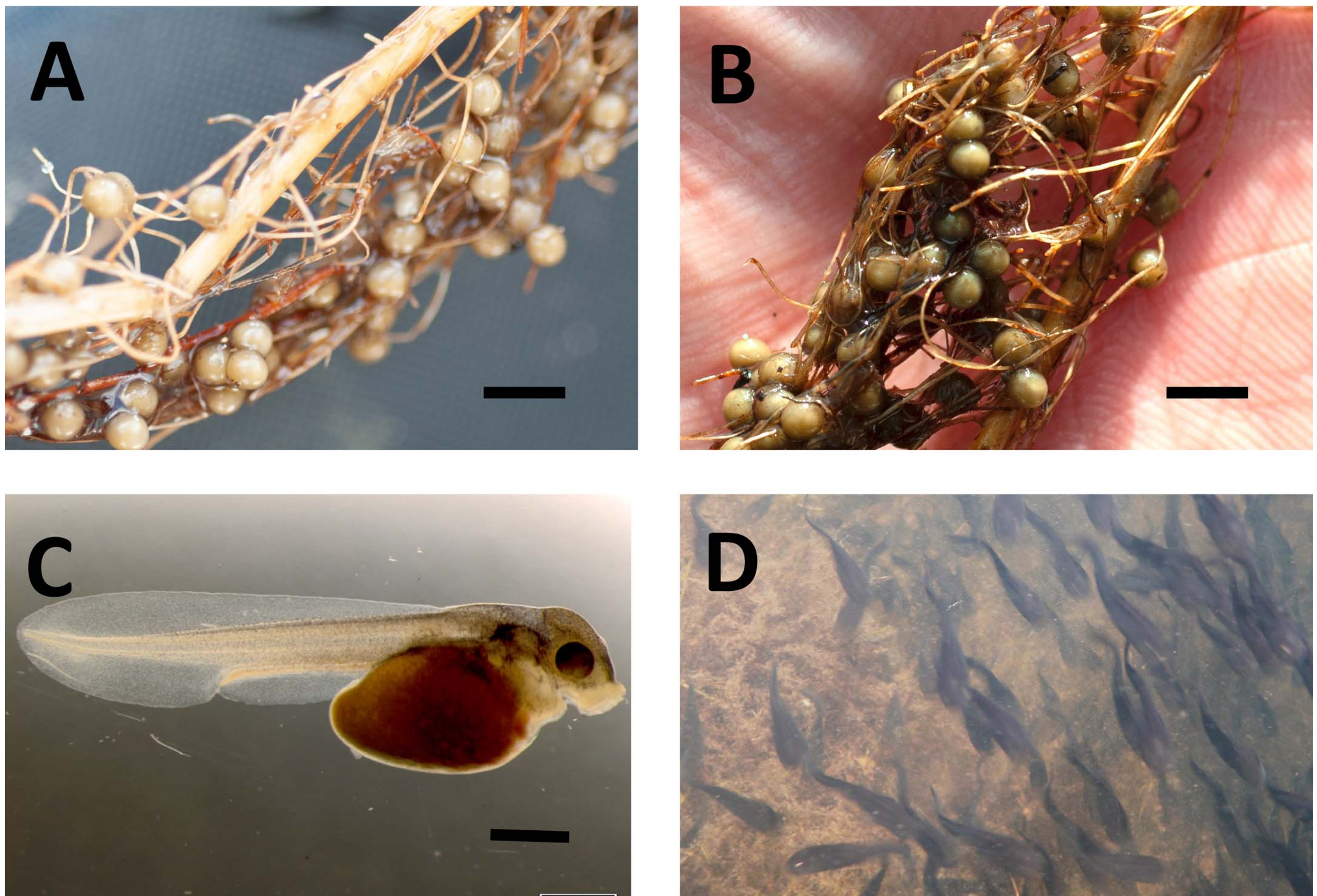

Fig. 2. Bowfin eggs, larva, and fry from Big Bay Creek, New York. (A) Freshly laid bowfin eggs are about 2.2 mm in diameter and white for about the first 24 hrs (photo by Emily Funk using a Nikon D90 camera; scale bar = 5 mm). (B) After about 24 hours, eggs turned greenish gray and are more cryptic (photo by Emily Funk using a Nikon D90 camera; scale bar = 5 mm). (C) Bowfin larva, 7.8 mm long, which is largely translucent except for yolk, eyes, and early pigmentation (photo by Emily Funk using a DP25 camera fitted to an Olympus SZX16 dissection microscope; scale bar = 1 cm). (D) Fully black, free-swimming fry in fry ball. Fry are approximately 40 mm in length (underwater photo by A. McCune using an Olympus Stylus Tough 8000 camera).

buffer (Life Technologies), 2 mM $MgCl_2$, 0.2 mM dNTPs, 2 picomoles each primer, 0.1 μL Platinum Taq, and 1 μL bowfin genomic DNA. Reactions employed a "touchdown" cycling protocol of 94°C for 50 seconds, 62°C–56°C for 45 seconds (dropping the annealing temperature one degree per cycle for the first seven cycles), 72°C for 1.5 minutes, followed by 28 cycles of 94°C for 50 seconds, 55°C for 45 seconds, 72°C for 1.5 minutes, with an 18°C hold.

A fragment of the bowfin mitochondrial D-loop was PCR-amplified from the 18 males guarding nests with primers 5′–CTCGGCTCCCAAAGCTGAGATTTT–3′ and 5′–TGTTGTC GTGAATTTTATGGGTATAATTCACGG–3′. The composition and cycling of these PCRs was as described above for COI, although at somewhat higher annealing temperatures and two fewer cycles overall (64°C–59°C for the first six cycles, 57°C for the remaining 28 cycles).

PCR products to be sequenced were enzymatically treated with 0.1 μL Antarctic phosphatase and 0.1 μL Exonuclease 1 (New England Biolabs, Beverly, MA) in 5 μL 1x Platinum Taq buffer. Samples were incubated at 37°C for 45 minutes, then 90°C for 10 minutes. Aliquots of these treated PCR products were sequenced with BigDye version 3.1 cycle sequencing kits (Life Technologies) and one of the primers used in the original PCR reaction. Sequence data was collected with 3100 and 3730xl DNA Analyzers (Life Technologies).

Roche/454 sequencing of a bowfin genomic library enriched for microsatellite DNA.—A bowfin genomic DNA library was enriched for a set of dimeric, trimeric, and tetrameric repeats and sequenced with Roche/454 technologies (Andres and Bogdanowicz, 2011). Briefly, approximately 500 nanograms of genomic DNA was digested with *Hinc II* and ligated to a double-stranded adapter in the presence of *Pme I*, to prevent self-ligation of the linker. Adapter-ligated DNA was hybridized to 3′-biotinylated repeat oligonucleotides (representing two dimers, five trimers, and five tetramers), captured with streptavidin-coated magnetic beads (New England Biolabs [NEB], Beverly, MA), and made double-stranded by PCR with an adapter primer. PCR products were pooled and ligated to a Roche/454 Rapid Library adapter, and small fragments were removed with Ampure beads and a sizing solution (8.4% PEG-8000, 1.2 M NaCl, NEB protocol). Sequence data were collected on a Roche/454 platform with Titanium reagents and software. Reads were trimmed of adapter sequence and high-quality reads were assembled with SeqMan Pro software (DNASTAR, Madison, WI).

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Table 1. Sampled males and associated broods by date. Fin clips from males, paired with a sample of fry the male was guarding, have been deposited in the Cornell University Museum of Vertebrates (CUMV). We were not able to catch an associated male in two cases, a free-swimming school (denoted as X) and on nest #2012–13.

Date sampled	Male fin clip	Male TL	Offspring genotyped (*n*)	CUMV #
28 April 2009	21	not measured	30 fry	99934
12 May 2009	23	60 cm	30 fry	99839
12 May 2009	24	63 cm	27 fry	99840
13 May 2009	27	56 cm	30 eggs	99842
13 May 2009	28	44 cm	30 fry	99844
13 May 2009	29	56 cm	30 fry	99843
17 May 2009	31	61 cm	30 fry	99847
25 May 2011	54	not measured	31 fry	99935
30 May 2011	X	not caught	20 fry	99841
9 May 2012	59	58 cm	31 fry	99833
18 May 2012	60	58 cm	31 fry	99834
18 May 2012	61	60 cm	30 fry	99835
18 May 2012	62	61 cm	30 fry	99836
18 May 2012	63	57 cm	30 fry	99936
18 May 2012	13	not caught	30 fry	99832

Microsatellite primer design and testing loci.—PCR primers for genotyping were designed with PrimerSelect software (DNASTAR). We employed three-primer PCR to determine whether loci amplified cleanly and were polymorphic. One of the locus-specific primers was tagged at the 5′ end with a 20 bp "long tag" (5′–CGAGTTTTCCCAGTCACGAC–3′ Schuelke, 2000), the other primer was modified with a 5′ "pig tail" (5′–GTTTCTT–3′; Brownstein et al., 1996). For three-primer PCR, each pair of locus-specific primers was mixed with a third primer identical to the long tag sequence and with a 5′-6FAM dye. The PCR concentrations of locus-specific pig-tailed and long tag primers was 0.2 μM and 0.05 μM, respectively, while the concentration of the 6FAM long tag was 0.15 μM. PCR reaction volume was 10 μL, and cycling consisted of 35 cycles at 94°C for 50 seconds, 54°C for 45 seconds, 72°C for 1 minute. The resulting PCR products were analyzed first on 1% agarose gels; clean/robust PCR products were then analyzed on ABI 3100 or 3730xl capillary sequencers to determine levels of polymorphism. Loci used for genotyping had the previously long-tagged primer resynthesized with a 5′-labeled fluorescent dye compatible with the G5 dye set (Life Technologies, Carlsbad, CA). The long tag was omitted from these 5′ dye-labeled primers.

Microsatellite DNA genotyping.—Two primer mixes were constructed for multiplex PCR using a Type-It Microsatellite PCR kit (Qiagen, Valencia, CA). Primer mix one contained six loci (five tetrameric repeats and one trimeric repeat) and mix two contained five loci (two trimeric repeats and three dimeric repeats; Table 1). Each adult male or fry DNA sample analyzed was amplified for each primer mix (11 loci total). PCR reaction volume was 10 μl and consisted of 1 μL of DNA, 3 μL of molecular biology grade (MBG) water, 5 μL of 2x Type-It master mix, and 1 μL of primer mix 1 or 2. Reactions were heated at 95°C for 5 minutes, then cycled 27 times at 94°C for 50 seconds, 55°C for 90 seconds, and 72°C for 30 seconds, followed by 30 minutes at 60°C. PCR products were diluted 1:30 in MBG water, and 1.5 μL of each diluted PCR product was combined with 15 μL Hi-Di Formamide and 0.12 μL Genscan LIZ-500 ladder (both from Life Technologies). Data were collected on ABI 3100 and 3730xl capillary sequencers. Allele calls were made with Genemapper v. 4.0 software (Life Technologies). We used CERVUS v. 3.0.3 (Kalinowski et al., 2007) to calculate allele frequencies and probabilities of both identity and parental exclusion for our population sample (excluding fry).

Ascertainment of parentage and polygamy.—We used the program CERVUS v. 3.0.3 (Kalinowski et al., 2007) to answer the question: is the multilocus microsatellite DNA genotype displayed by the adult male guarding each brood consistent with that male being a parent of each of the genotyped fry he was guarding? To infer the genotype of the primary spawning female, we first assumed that the most abundant fry genotypes in a nest represent the guarding male and inferred primary female. By subtraction, we could infer the primary female's genotype from the combination of fry genotypes, given the male genotype. We refer to the guarding male and the inferred primary female as the primary pair. We used simple Mendelian inheritance to determine cases of polygamy, in which one or more extra-pair (EP) adults contributed alleles present in some of the offspring in a brood, but these alleles were not attributable to either of the primary pair. We compared the genotypes of each of the sampled fry at a nest and the male guarding them, for each microsatellite locus. Polygamy was invoked when we observed more than two diploid genotypic classes at any microsatellite locus among offspring when the adult male was a homozygote at that locus, when we observed more than four genotypic classes among fry when the adult male was heterozygous at a given locus, and/or if there were fry genotypes inconsistent with the genotypes of the guarding male and inferred primary female. We inferred polyandry when a guarded fry's genotype did not contain either of the guarding male's alleles at a locus. Polygyny was indicated when a fry's genotype did not contain either of the inferred female alleles at a locus and/or when a series of fry from the same nest exhibited variation in size of the mitochondrial D-loop PCR fragment. In the two broods for which we lacked the guarding male genotype, we were only able to infer polygamy without specifying whether polygyny or polyandry was indicated. In two cases, there were single fry

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Table 2. Microsatellite DNA summary statistics. Reported characteristics at each locus are based on the population sampled of adults from Big Bay Creek, Oneida Lake, New York: number of alleles (k), observed (H_O) and expected (H_E) heterozygosities, and inferred frequencies of null alleles (Null). Reverse (bottom) primers at each locus have a GTTTCTT "pig tail" at the 5′ end (Brownstein et al., 1996).

Locus	Repeat	Primers (5′–3′)	k	H_O	H_E	Null
6di	AG	TGATTGAATATGAAACGCTGGTAG *GTTTCTT*ATTGGGTGCCACAGTTCATCT	4	0.259	0.309	0.091
9di	AG	ACAGCCCCAAATCCTTATCCATT *GTTTCTT*AGGCGCCCTCGCTATCTGT	4	0.588	0.663	0.063
20di	AG	AGCACATGCCCTCAATACATAACT *GTTTCTT*AATACAGGGTGGTTTGTTTGTCAG	3	0.635	0.641	0.005
48tri	AAC	TCAAAGCAAAAGGATTGCAGAA *GTTTCTT*TCCGGTATGGCAAAGCAAGAC	2	0.235	0.244	0.015
133tet	AGAT	CTGCTTAAAATGATCGCCTTGAC *GTTTCTT*AGCCTTTTGGGGTTTGTGC	4	0.682	0.680	0.007
325tet	AAAG	CACCGCACAATAGCAACTCCATA *GTTTCTT*CGTCCCGAACCCCTACAATG	3	0.329	0.389	0.079
5001tet	AGAT	ACATCAAAACGTTGCCTGTGTTT *GTTTCTT*TTTTGTCGGAAGCCTTCATCC	3	0.635	0.636	0.004
10875tet	AGAT	GACTGCGTGGAACGAACTGC *GTTTCTT*ACGCTGTCTTGCCTTTACACTGT	3	0.494	0.580	0.085
14619tet	AGAT	TGCCTTCAATTAAGACACAACGAG *GTTTCTT*CCCTTCTACGGCTTGGAAAGA	2	0.235	0.261	0.048
15194tri	ACT	GACCTGGAGAAGAGCGTCTGC *GTTTCTT*GTTGAGGAGCGTTTCTATGGTTGT	4	0.318	0.323	0.041
16306tri	AAC	TTTGCCTGTCATGGGGTCAATAC *GTTTCTT*TTGGCGTTCACCGAATACGAG	6	0.600	0.687	0.080

at a single locus with a genotype not explained by the parent genotypes, which we regard as insufficient evidence for polygamy given possible explanations for such a rare genotype (including genotyping error, orphan fry, or germline mutation). For the subset of broods with an identified paternal genotype, we estimated the minimum number of additional parents contributing genes to a brood by counting the number of alleles represented in the brood which were not also represented in the primary spawning pair and determining the minimum number of EP adults required to account for these extra alleles. This is a minimum estimate because both alleles of an extra parent may not be represented in the brood and there may be extra adults with alleles identical to those of the primary spawning pair.

RESULTS

Mitochondrial COI and D-loop PCR and sequencing.—Our 1,315 base-pair COI sequences showed 99–100% identity to published COI sequences of *Amia* (accession numbers AB042952.1 and AY442347.1, both complete mtDNA genomes of *Amia*, and EU523910.1, EU524434.1, and EU524435.1, COI sequences of *Amia*) but were completely identical among the 24 lake-wide adults sequenced. Given the apparent lack of variation at this locus in Oneida bowfin, we did not use COI in any further analyses.

Mitochondrial D-loop PCRs yielded fragments that were much more complex and variable in size than COI. We estimate there are at least six different size classes at this locus among *Amia* from Oneida Lake, ranging in size from approximately 400–1,200 bp. The D-loop PCR fragment often presents as a smear (rather than a discrete fragment) upon agarose gel electrophoresis. The two published and complete *Amia* mitochondrial genomes (see accession numbers in the previous paragraph) differ by about 780 bp; virtually all of that difference in size is driven by size differences in the D-loop, which is highly repetitive in both complete mitochondrial genomes.

Microsatellite DNA: summary statistics and parental exclusion probabilities.—Eight samples from the lake-wide survey genotyped poorly and were removed from these analyses. Microsatellite loci among Oneida Lake *Amia* are moderately variable, with 2–6 alleles per locus (mean 3.45 alleles/locus) and expected heterozygosities ranging from 0.244–0.687 (Table 2). Combined non-exclusion probabilities for the first parent, second parent, and parental pair are 0.173, 0.029, and 0.003, respectively. Table 3 shows allele frequencies and polymorphic information content (PIC) at each locus.

Microsatellite DNA: evidence of monogamy and polygamy.—For seven broods of *Amia* out of 15 (47%) sampled in waters connected to Oneida Lake there is essentially no evidence of polygamy, suggesting that the offspring in each of those nests were derived from monogamous spawnings (see Table 4). In two of these nests (54, 61), however, a single fry at a single microsatellite locus could not be ascribed to the same male–female pair as the rest, making it difficult to say definitively whether mating was monogamous or polygamous. A single oddball fry from one locus such as this might be due to insufficient sampling of offspring, an orphan fry mixed into the brood, a genotyping error, or even a germline mutation.

Eight broods (associated with males 27, 29, 31, 59, 60, 61, male X and nest 2012–13), showed clear evidence of genetic polygamy (Table 5), minimally one or two EP adults being involved in spawning in addition to the primary pair. For

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Table 3. Allele sizes, allele frequencies, and polymorphic information content (PIC) at 11 microsatellite DNA loci. Adult bowfin (n = 85) sampled from Oneida Lake, New York. Allele sizes are in base pairs.

Locus	Allele size	Allele freq.	PIC
6di	308	0.012	0.276
	310	0.153	
	318	0.818	
	320	0.017	
9di	286	0.406	0.594
	288	0.147	
	294	0.388	
	298	0.059	
20di	336	0.382	0.560
	338	0.424	
	342	0.194	
48tri	244	0.859	0.213
	259	0.141	
133tet	329	0.159	0.615
	337	0.400	
	341	0.365	
	345	0.076	
325tet	277	0.253	0.316
	281	0.741	
	285	0.006	
5001tet	158	0.371	0.556
	170	0.441	
	174	0.188	
10875tet	219	0.124	0.499
	231	0.547	
	247	0.329	
14619tet	278	0.847	0.226
	282	0.153	
15194tri	284	0.006	0.283
	287	0.017	
	290	0.171	
	293	0.806	
16306tri	245	0.041	0.643
	254	0.488	
	257	0.106	
	260	0.224	
	263	0.018	
	269	0.123	

five of these broods, genotypes of offspring show evidence of polygyny, with one or two additional females spawning in each nest (nests of males 27, 29, 31, 59, and 60). Offspring associated with male 29 also showed variation in D-loop (Table 6), which might be expected to occur in broods with more than one dam. Extra males were also occasionally involved in spawning, with clear evidence of polyandry also found in two of the polygynous broods (27, 31) as well as among the fry being guarded by male 61. In the two nests for which we lacked male tissue (male X and nest 2012–13), we were unable to determine whether polygamy was a case of polygyny or polyandry. Overall, we found seven clear cases of genetic monogamy and eight clear cases of polygamy. Among the polygamous broods, we identified five broods showing evidence of genetic polygyny and three broods showing evidence of genetic polyandry.

Across all broods, the minimum number of EP adults participating in spawning was 0–2 (Table 7). Note that this is a minimum estimate, as described in the methods and in the caption for Table 7 because our method cannot distinguish two EP adults each contributing one allele from a heterozygous EP adult contributing both alleles. In all broods but one, fewer than 30% of the offspring carried one or more alleles from an EP adult. Male 31, however, was guarding a brood in which 50% of the offspring carried an allele from an EP adult (Table 7).

DISCUSSION

Bowfins are widely regarded as promiscuous or polygamous (e.g., Gill, 1905, p. 432; Breder and Rosen, 1966, p. 70; Smith, 1985; Jenkins and Burkhead, 1994; Burr and Bennett, 2014) following Reighard's (1903, p. 102) conjecture that more than one female might spawn in the same nest with the same male and that a female bowfin might spawn in more than one nest with more than one male. Our data do not address whether an individual female will spawn in more than one nest because we were not able to genotype females. However, our study demonstrates that Reighard was correct that multiple females may spawn in a single nest. In at least four cases, a guarding male was able to attract at least 1–2 additional females to spawn in his nest. Our evidence of polygyny derives from two distinct genetic markers: microsatellite DNA and mitochondrial D-loop sequences (nest 29, see Table 6). Assuming mitochondrial DNA is maternally inherited in *Amia*, the D-loop size variation we observe in the fry from nest 29 is further evidence that multiple females spawned in that nest. This makes sense as discussed by more recent authors: an individual male able to attract multiple females to lay eggs in his nest may leave more offspring (Emlen and Oring, 1977; Turner, 1986; Davies and Krebs, 2012; Rueger et al., 2019). To categorize *Amia* as simply genetically polygynous, however, would overlook interesting aspects of their reproductive natural history.

To our knowledge, no one has suggested explicitly that bowfins are monogamous, although behavioral monogamy was what Reighard described: the male attracts a female to his nest and spawns with her one or more times (Reighard, 1903). Observed spawning behavior is thus consistent with behavioral monogamy, and our genetic data show that nearly half of the broods in our sample are derived from monogamous mating of the guarding male with a single female. For bowfin, like many socially monogamous vertebrates, there can be an extra-pair gametic contribution to the offspring in a nest even though they appear to be monogamous (DeWoody and Avise, 2001; Avise et al., 2002; Whiteman and Côté, 2004; Rueger et al., 2019), as explained above. Note that we describe bowfin mating as behaviorally monogamous rather than socially monogamous because we do not mean to imply a long-term social relationship that could be implied by the term social monogamy.

Polyandry also does not seem to have been suggested previously for bowfins. Our data indicate that one additional male sired offspring in three nests (Table 5), apparently able to avoid the well-known aggressive behavior of guarding bowfin males (e.g., Reighard, 1903, p. 84). In two of these cases, an extra-pair (EP) male contributed to less than 3% of sampled offspring in the nest. However, in one case, an EP male sired as many as 50% of the offspring. Thus, in 93% of broods, fewer than 3% of offspring were sired by an EP male, but in 7% of broods, an EP male fertilized many eggs. Polyandry is clearly not a fixed strategy in bowfins, but, at

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Table 4. Evidence of monogamous spawning. Monogamy is assumed when no excess genotypes are represented in fry relative to expectation, given the genotypes of the guarding male (GM) and the inferred primary female (IPF). To infer the genotype of the IPF, we first assumed that the most abundant fry genotypes represent the GM and IPF. If the GM is homozygous, we expect no more than two distinct diploid genotypes represented among the fry. If the GM is heterozygous, we expect no more than four distinct diploid genotypes represented among the fry. Furthermore, for the monogamous case, fry genotypes must be consistent with the genotypes of the GM and IPF. To recognize excess genotypes, we used a threshold of greater than one fry genotype at one locus, not explained by the genotypes of the GM and IPF, because categorizing a spawning event as polygamous based on only a singleton fry at only one of 11 loci would be very weak evidence of polygamy. Alternative explanations for a single unexplained fry genotype, given the parents, include an orphan fry joining a brood, a genotyping error, or a germline mutation.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Inferred mating system (at locus)
21	5001_tet	158/178	158/178	13	158/170	Monogamy
			170/178	11		
			158/170	6		
			158/158	4		
	10875_tet	247/247	247/247	18	219/247	Monogamy
			219/247	16		
	16306_tri	254/269	254/269	14	254/269	Monogamy
			269/269	13		
			254/254	7		
	48_tri	244/259	244/244	18	244/244	Monogamy
			244/259	16		
	14619_tet	278/278	278/278	34	278/278	Monogamy
	325_tet	277/277	277/281	34	281/281	Monogamy
	15194_tri	290/293	293/293	18	293/293	Monogamy
			290/293	16		
	133_tet	333/345	337/345	19	337/337	Monogamy
			333/337	15		
	6_di	318/320	318/320	18	318/318	Monogamy
			318/318	16		
	9_di	294/294	288/294	34	288/288	Monogamy
	20_di	336/342	336/342	18	336/336	Monogamy
			336/336	16		
23	5001_tet	158/170	158/170	18	158/170	Monogamy
			170/170	11		
			158/158	7		
	10875_tet	219/219	219/247	36	247/247	Monogamy
	16306_tri	254/257	254/254	19	254/254	Monogamy
			254/257	17		
	48_tri	244/259	244/244	20	244/244	Monogamy
			244/259	16		
	14619_tet	278/278	278/278	36	278/278	Monogamy
	325_tet	277/281	281/281	23	281/281	Monogamy
			277/281	13		
	15194_tri	290/293	293/293	20	293/293	Monogamy
			290/293	16		
	133_tet	337/345	341/345	12	337/341	Monogamy
			337/345	9		
			337/341	8		
			337/337	7		
	6_di	318/318	318/318	36	318/318	Monogamy
	9_di	286/294	286/294	12	286/288	Monogamy
			286/288	10		
			286/286	8		
			288/294	6		

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Table 4. Continued.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Inferred mating system (at locus)
	20_di	336/336	336/336	36	336/336	Monogamy
24	5001_tet	158/174	170/174	12	170/174	Monogamy
			158/170	10		
			158/174	9		
			174/174	5		
	10875_tet	231/231	231/247	36	247/247	Monogamy
	16306_tri	254/263	260/263	20	260/260	Monogamy
			254/260	16		
	48_tri	244/244	244/259	20	244/259	Monogamy
			244/244	16		
	14619_tet	278/278	278/278	36	278/278	Monogamy
	325_tet	281/281	277/281	36	277/277	Monogamy
	15194_tri	293/293	284/293	20	284/293	Monogamy
			293/293	16		
	133_tet	337/345	337/337	23	337/337	Monogamy
			337/345	13		
	6_di	318/318	310/318	36	310/310	Monogamy
	9_di	286/288	286/288	23	286/286	Monogamy
			286/286	13		
	20_di	336/338	338/338	10	338/342	Monogamy
			336/342	10		
			336/338	8		
			338/342	8		
28	5001_tet	170/174	170/170	11	158/170	Monogamy
			170/174	10		
			158/170	8		
			158/174	7		
	10875_tet	231/247	231/247	13	219/247	Monogamy
			219/231	11		
			247/247	7		
			219/247	5		
	16306_tri	260/269	260/269	16	260/269	Monogamy
			260/260	11		
			269/269	9		
	48_tri	244/259	244/244	18	244/244	Monogamy
			244/259	18		
	14619_tet	278/282	278/278	20	278/278	Monogamy
			278/282	16		
	325_tet	281/281	281/281	18	277/281	Monogamy
			277/281	18		
	15194_tri	290/293	293/293	18	293/293	Monogamy
			290/293	18		
	133_tet	341/341	341/345	20	341/345	Monogamy
			341/341	16		
	6_di	310/310	310/318	35	318/318	Monogamy
	9_di	286/294	286/294	17	286/294	Monogamy
			286/286	11		
			294/294	8		

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Table 4. Continued.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Inferred mating system (at locus)
	20_di	338/342	336/338	15	336/342	Monogamy
			342/342	10		
			338/342	7		
			336/342	4		
54	5001_tet	170/174	158/170	19	158/158	Monogamy
			158/174	12		
	10875_tet	231/247	247/247	17	247/247	Monogamy
			231/247	14		
	16306_tri	254/257	257/269	18	269/269	Monogamy
			254/269	13		
	48_tri	244/244	244/244	31	244/244	Monogamy
	14619_tet	278/278	278/278	31	278/278	Monogamy
	325_tet	277/281	277/281	18	277/281	Monogamy
			281/281	9		
			277/277	4		
	15194_tri	290/293	290/293	10	287/293	Monogamy
			293/293	9		
			287/290	7		
			287/293	4		
			287/296	1		
	133_tet	329/341	329/341	9	333/341	Monogamy
			329/333	9		
			333/341	8		
			341/341	5		
	6_di	310/318	310/320	14	318/320	Monogamy
			318/320	9		
			318/318	6		
			310/318	2		
	9_di	286/294	294/294	11	284/294	Monogamy
			286/294	9		
			284/294	6		
			284/286	5		
	20_di	336/338	336/342	9	338/342	Monogamy
			338/342	9		
			338/338	7		
			336/338	6		
62	5001_tet	170/174	158/170	16	158/158	Monogamy
			158/174	14		
	10875_tet	231/247	231/247	16	231/247	Monogamy
			247/247	9		
			231/231	4		
	16306_tri	254/254	254/257	19	254/257	Monogamy
			254/254	11		
	48_tri	244/244	244/244	30	244/244	Monogamy
	14619_tet	278/278	278/278	30	278/278	Monogamy
	325_tet	277/281	277/281	16	281/281	Monogamy
			281/281	14		
	15194_tri	293/293	293/293	30	293/293	Monogamy

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Table 4. Continued.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Inferred mating system (at locus)
	133_tet	337/341	337/341	13	337/341	Monogamy
			337/337	9		
			341/341	8		
	6_di	310/310	310/318	30	318/318	Monogamy
	9_di	286/288	288/294	11	286/294	Monogamy
			286/294	8		
			286/288	6		
			286/286	5		
	20_di	336/338	336/336	18	336/336	Monogamy
			336/338	12		
63	5001_tet	170/170	158/170	18	158/170	Monogamy
			170/170	12		
	10875_tet	219/247	219/247	17	219/247	Monogamy
			219/219	7		
			247/247	6		
	16306_tri	259/259	254/259	17	254/269	Monogamy
			259/269	12		
			257/259	1		
	48_tri	244/244	244/244	30	244/244	Monogamy
	14619_tet	278/278	278/278	30	278/278	Monogamy
	325_tet	277/281	277/281	16	281/281	Monogamy
			281/281	14		
	15194_tri	293/293	293/293	30	293/293	Monogamy
	133_tet	341/341	341/341	30	341/341	Monogamy
	6_di	310/310	310/318	30	318/318	Monogamy
	9_di	286/298	286/288	15	288/288	Monogamy
			288/298	15		
	20_di	336/336	336/336	17	336/342	Monogamy
			336/342	13		

the same time, an EP male can sometimes be very successful in siring offspring that will then be guarded by other males. These genetic data are consistent with Dean's (1898) earlier observation of three fish (not identified as male or female) seen on a nest during spawning, although the observation was suggested as evidence for group spawning and previously dismissed as an "abnormality" (Breder and Rosen, 1966). Reighard (1903) had also observed additional males "lurking" or "swirling" near a nest with a spawning pair but viewed this as evidence that males were simply aware of the presence of females. In retrospect, it seems likely that these lurking males might have been seeking an opportunity to spawn. Reighard did not note whether the "lurking males" were smaller than the spawning males, as might be expected of sneaker males.

With three clear cases of EP males contributing genes to offspring being guarded by other males, further study is merited to clarify the circumstances in which those EP males participate in spawning. Is there occasional participation by sneaker males? Or might our data reflect incidental contamination from adjacent nests? Clearly, there is still much to be learned about bowfin reproductive ecology. With infrared cameras, it might be possible to observe spawning at night. Given the apparent site fidelity of some individuals during and after spawning (J. R. Jackson and A. R. McCune, unpubl. telemetry data), it should be possible to make a large, localized tagging study to obtain genotypes of spawning females as well as track their movements in, around, and out of the spawning grounds. With our initial genetic data showing complexity of parentage, the quality of sampling could be maximized by collecting mobile fry (rather than eggs or immobile larvae) to ensure that fry from different spawnings in that nest are well mixed and thus ameliorate any potential spatial pattern of fertilized eggs that might result from different females spawning in different areas of the nest.

An alternative explanation for excess genotypes represented in offspring at some nests is that males could be caring for abandoned fry from other spawning pairs. Given that the nests are well defined and tenaciously guarded, it seems somewhat unlikely that eggs or non-swimming larvae would get mixed up. However, once the fry are mobile, this

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Table 5. Evidence of polygamous spawning. Evidence of polygamy among bowfin broods is based on excess numbers of genotypes represented by fry, given the genotype of the guarding male (GM). If the GM is homozygous, we infer polygamy if there are more than two distinct diploid genotypes represented among the fry. If the GM is heterozygous, we infer polygamy if there are more than four distinct diploid genotypes represented among the fry and/or if there are fry genotypes inconsistent with the genotypes of the GM and inferred primary female (IPF). In most cases, we were able to infer whether excess genotypes originated from extra females or males. To do so, we first assumed that the most abundant fry genotypes are derived from spawning of the GM and IPF. Polyandry is indicated if there are excess fry genotypes lacking an allele from the GM. Polygyny is indicated if there are fry genotypes lacking an allele from the IPF. All fry genotypes inferred to be offspring from a non-primary spawner are shown in italics. Fry genotype combinations demonstrating polyandrous mating are also shown in bold. Fry genotype combinations demonstrating polygynous mating are underlined. For the two cases where we were unable to capture the guarding male (X and at nest 2012–13), we infer polygamy if there are more than four diploid genotypes represented among the fry.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Extra parents inferred from excess genotypes	Inferred mating system (from locus)
27	16306_tri	260/260	260/245	20	245/254		
			260/254	12			
			260/260	1		*Extra F*	polygyny
			260/269	1		*Extra F*	and
			254/254	1		*Extra M*	polyandry
			254/245	1		*Extra M*	
	20_di	342/342	342/342	19	338/342		
			342/338	14			
			342/336	3		*Extra F*	polygyny
	5001_tet	158/170	158/170	10	174/170		
			158/174	10			
			170/170	7			
			170/174	6			
			158/158	3		*Extra F*	polygyny
	10875_tet	219/231	231/247	21	247/247		
			219/247	12			
			231/231	2		*Extra F*	polygyny
			219/219	1		*Extra F*	
	133_tet	337/341	337/341	13	329/337		
			337/337	9			
			337/329	6			
			341/329	6			
			341/341	2		*Extra F*	polygyny
	9_di	288/294	294/286	15	286/294		
			288/286	14			
			288/294	4			
			294/294	1			
			286/298	1		*Extra M*	polyandry
			286/286	1		*Extra M*	
29	20_di	336/336	336/336	15	336/338		
			336/338	15			
			336/342	6		*Extra M or F*	polygamy
	16306_tri	254/257	257/254	9	254/260		
			254/254	8			
			257/260	6			
			254/260	4			polygyny with 2 EPs, F
			257/257	5		*Extra F*	is most parsimonius
			254/269	2		*Extra M or F*	
			257/263	1		*Extra F*	
			257/269	1		*Extra F*	
	133_tet	337/341	337/341	10	329/341		
			341/341	7			
			337/329	5			
			341/329	4			

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Table 5. Continued.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Extra parents inferred from excess genotypes	Inferred mating system (from locus)
			337/345	5		*Extra F*	polygyny
			341/345	3		*Extra M or F*	is most parsimonius
			337/337	2		*Extra F*	
	9_di	286/288	286/288	12	288/294		
			286/294	8			
			288/288	7			
			288/294	3			
			286/286	6		*Extra F*	polygyny
31	10875_tet	231/231	231/247	16	231/247		
			231/231	6			
			231/219	5		*Extra M or F*	
			219/219	4		*Extra M and F*	polygyny
			219/247	4		*Extra M*	and
			247/247	1		*Extra M*	polyandry
	16306_tri	254/254	254/269	20	269/254		
			254/254	5			
			254/260	4		*Extra M*	polyandry
			269/260	3		*Extra M*	and
			269/269	1		*Extra M*	polygamy
			254/245	1		*Extra M or F*	
	133_tet	329/337	337/341	13	337/341		
			329/341	10			
			337/337	6			
			329/337	1			
			337/345	3		*Extra M or F*	polygamy
			337/334	2		*Extra M or F*	and
			341/341	1		*Extra M*	polyandry
	9_di	286/286	286/294	10	286/294		
			286/286	8			
			286/288	4		*Extra M or F*	
			288/294	4		*Extra M*	polyandry
			294/294	4		*Extra M*	
			286/298	2		*Extra M or F*	and
			288/298	2		*Extra M and F*	polygyny
			288/288	1		*Extra M and F*	
			294/298	1		*Extra M*	
	20_di	336/336	336/336	17	336/338		
			336/338	11			
			336/342	4		*Extra M or F*	polygyny
			342/342	4		*Extra M and F*	polyandry
59	16306_tri	245/254	254/254	13	254/254		
			245/254	14			
			254/268	2		*Extra F*	polygyny
			245/259	1		*Extra F*	
			245/268	1		*Extra F*	
	133_tet	329/341	337/341	13	337/337		
			329/337	16			
			329/343	1		*Extra F*	polygyny
			341/343	1		*Extra F*	
60	5001_tet	158/170	158/170	13	158/170		
			170/170	7			
			158/158	5			
			158/173	3		*Extra M or F*	polygamy
			170/173	2		*Extra M or F*	

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Table 5. Continued.

Nest	Locus	GM genotype	Fry genotypes	*n*	IPF genotype	Extra parents inferred from excess genotypes	Inferred mating system (from locus)
	16306_tri	254/259	254/254	11	254/268		
			259/254	9			
			259/268	4			
			254/268	3			
			259/259	3		*Extra F*	polygyny
	20_di	336/336	336/336	11	336/338		
			336/338	10			
			336/342	9		*Extra M or F*	polygamy
61	5001_tet	170/170	170/170	16	158/170		
			158/170	13			
			158/174	1		*Extra M*	polyandry
	325_tet	277/277	277/281	29	281/281		
			281/281	1		*Extra M*	polyandry
2012–13	133_tet	unknown	341/329	10	unknown		
			337/329	7			
			341/341	6			
			341/337	3			
			345/329	2		*Extra M or F*	polygamy
			337/337	1		*Extra M or F*	
			341/345	1		*Extra M or F*	
	9_di	unknown	298/294	9	unknown		
			294/288	6			
			286/288	6			
			298/288	4			
			298/298	2		*Extra M or F*	polygamy
			298/286	1		*Extra M or F*	
			294/290	1		*Extra M or F*	
X	10875_tet	unknown	231/247	7	unknown		
			231/231	6			
			247/247	4			
			219/247	2			
			219/231	1		*Extra M or F*	polygamy
	16306_tri	unknown	254/260	9	unknown		
			254/254	3			
			260/260	4			
			254/269	2			
			260/257	1		*Extra M or F*	polygamy
			260/269	1		*Extra M or F*	
	133_tet	unknown	337/341	7	unknown		
			341/341	6			
			329/341	5			
			345/345	1			
			337/337	1		*Extra M or F*	polygamy
	9_di	unknown	286/298	5	unknown		
			286/286	4			
			286/288	4			
			288/298	2			
			286/294	3		*Extra M or F*	polygamy
			298/298	1		*Extra M or F*	
			288/288	1		*Extra M or F*	

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Table 6. Confirming evidence of polygyny from D-loop for nest 29. Fry in nest 29 exhibit two different mitochondrial D-loop lengths (500 and 1200 bp); inferred parental female genotypes for each of these size classes are based on alleles in excess of two which do not belong to the male for a particular locus.

	Locus								
Individual	16306tri		133tet		Di9		Di20		D-loop (bp)
Male	254	257	337	341	286	288	336	336	
Fry29_01	254	254	337	341	286	286	336	338	500
Fry29_02	257	269	329	337	286	294	336	342	1200
Fry29_03	254	257	337	341	286	286	336	336	500
Fry29_04	254	254	337	341	286	288	336	336	500
Fry29_05	254	260	337	341	288	288	336	336	500
Fry29_06	254	257	337	337	286	294	336	338	1200
Fry29_07	254	257	337	341	286	288	336	338	500
Fry29_08	257	257	329	341	288	294	336	338	1200
Fry29_09	254	257	329	341	288	294	336	342	1200
Fry29_10	257	260	337	345	286	288	336	336	500
Fry29_11	257	260	337	345	286	288	336	336	500
Fry29_12	254	257	337	341	286	286	336	338	500
Fry29_13	254	257	341	341	288	288	336	338	500
Fry29_14	254	254	341	341	288	288	336	336	500
Fry29_15	254	269	329	337	286	294	336	338	1200
Fry29_16	254	260	337	341	286	288	336	336	500
Fry29_17	254	254	341	341	286	288	336	338	500
Fry29_18	254	257	341	341	286	288	336	336	500
Fry29_19	257	260	337	345	286	288	336	338	500
Fry29_20	257	257	329	337	288	294	336	342	1200
Fry29_21	254	254	341	345	288	288	336	338	500
Fry29_22	254	254	341	341	286	288	336	336	500
Fry29_23	257	257	329	337	286	294	336	338	1200
Fry29_24	254	260	341	345	288	288	336	338	500
Fry29_25	254	257	329	341	286	294	336	342	1200
Fry29_26	254	254	337	341	286	288	336	336	500
Fry29_27	254	269	329	337	286	294	336	338	1200
Fry29_28	257	257	337	337	286	294	336	342	1200
Fry29_29	257	257	329	341	286	294	336	342	1200
Fry29_30	254	257	341	341	286	286	336	338	500
Fry29_31	254	254	337	341	288	288	336	338	500
Fry29_32	257	260	337	345	286	288	336	336	500
Fry29_33	257	260	341	341	286	286	336	336	500
Fry29_34	254	260	341	345	286	286	336	336	500
Fry29_35	257	263	337	345	286	288	336	336	500
Fry29_36	257	260	337	341	288	288	336	336	500
Inferred Female 1	*254*	*260*	*341*	*345*	*286*	*288*	*336*	*338*	*500*
Inferred Female 2	*257*	*269*	*329*	*337*	*294*	*294*	*338*	*342*	*1200*

seems more plausible. In one case described by Reighard (1903, p. 98), a male was guarding a traveling school of larger fry thought to have originated from two different nests as evidenced by a bimodal distribution of fry length. Males guarding unrelated fry is known in other fishes, such as lingcod, catfishes, and cichlids (Wisenden, 1999; Withler et al., 2004; Lee-Jenkins et al., 2015).

The finding that *Amia* is behaviorally monogamous, but also weakly genetically polygynous, is consistent with what has been learned from many genotyping studies in other vertebrates: socially monogamous species may not be genetically monogamous (DeWoody and Avise, 2001; Avise et al., 2002; Farias et al., 2015; Rueger et al., 2019). Among socially monogamous, nesting fishes with uniparental male care, the frequency of both multiple paternity and multiple maternity is highly variable (DeWoody and Avise, 2001; Coleman and Jones, 2011). At one end of the spectrum is the freshwater channel catfish, *Ictalurus punctatus*, which has been found to be both socially and genetically monogamous (Tatarenkov et al., 2006). More recently, genetic analysis has revealed even greater upper extremes of EP involvement in spawning among socially monogamous fishes. For example, 100% of analyzed broods of a socially monogamous osteoglossomorph, *Arapaima gigas*, involved EP contributions (Farias et al., 2015). In the temperate marine lingcod, *Ophiodon elongatus*, 70% of male-guarded nests contained eggs laid by a single female, but the eggs were fertilized by 2–5 males, making it clear that this species is genetically polyandrous (Withler et al., 2004).

Bowfin exhibit a lower level of both multiple maternity and paternity (in the 13 nests with EP participants identifiable by sex) in comparison to 23 other species of socially monogamous, male-guarded nesting fishes (Fig. 3) reviewed

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Table 7. Summary of mating system by nest as inferred from genotyped males and fry. Fin clips from males paired with a sample of fry they were guarding have been deposited in the Cornell University Museum of Vertebrates (Table 1). Polygamy noted here could be due to either accessory females or males. Polygyny and polyandry are indicated when the sex of an EP adult could be determined. The minimum number of extra-pair adults is inferred from the number of unique alleles that are not accounted for by the primary parents. The number could be higher if there are extra parents carrying the same alleles as parents and thus indistinguishable from them. The proportion of progeny in a brood derived from an EP adult is based on the locus with the highest proportion of offspring not accounted for by genotypes of primary parents (Table 5). We were not able to catch an associated male in two cases, a free-swimming school (denoted as X) and the GM for nest #2012–13.

Male fin clip	Offspring genotyped (*n*)	Inferred mating system	Minimum number of EP adults	% Progeny of EP males	% Progeny of EP females	% EP progeny total
21	30 fry	monogamy	0	0	0	0
23	30 fry	monogamy	0	0	0	0
24	27 fry	monogamy	0	0	0	0
27	30 eggs	polygyny, polyandry	2 (1F, 1M)	1%	10%	11%
28	30 fry	monogamy	0	0	0	0
29	30 fry	polygyny	2 (2F)	0	28%	28%
31	36 fry	polygyny, polyandry	2 (1F, 1M)	33–50%	8–25%	50%
54	31 fry	monogamy	0	0	0	0
X (no male)	20 fry	polygamy	1 (sex undet.)	–	–	25%
59	31 fry	polygyny	1 (1F)	–	13%	13%
60	31 fry	polygyny	1 (1F)	0	30%	30%
61	30 fry	polyandry	1 (1M)	3%	0	3%
62	30 fry	monogamy	0	0	0	0
63	30 fry	monogamy	0	0	0	0
2012–13 (no male)	30 fry	polygamy	1 (sex undet.)	–	–	13%

by Coleman and Jones (2011). Across these 23 species, an average of 76% of nests showed evidence of multiple maternity in contrast to the 33% of bowfin nests exhibiting multiple maternity. For this same set of 23 species, the average minimum number of females contributing eggs to a nest was 3.14 females, whereas for bowfin an average of 1.4 females contributed eggs to a nest. An average of 34% of nests across 23 species showed evidence of multiple paternity in comparison to 23% of 12 bowfin nests exhibiting multiple paternity. In these latter nests, the proportion of offspring sired by non-guarding males was quite low: 0–3% of offspring for all but one nest. In that nest, an EP male was unusually successful, siring as many as 50% of offspring.

When males provide uniparental care, rates of multiple paternity tend to be low compared to rates of multiple maternity (Coleman and Jones, 2011), and our results are consistent with this finding. However, as described above, the rates of multiple paternity and multiple maternity are overall relatively low in

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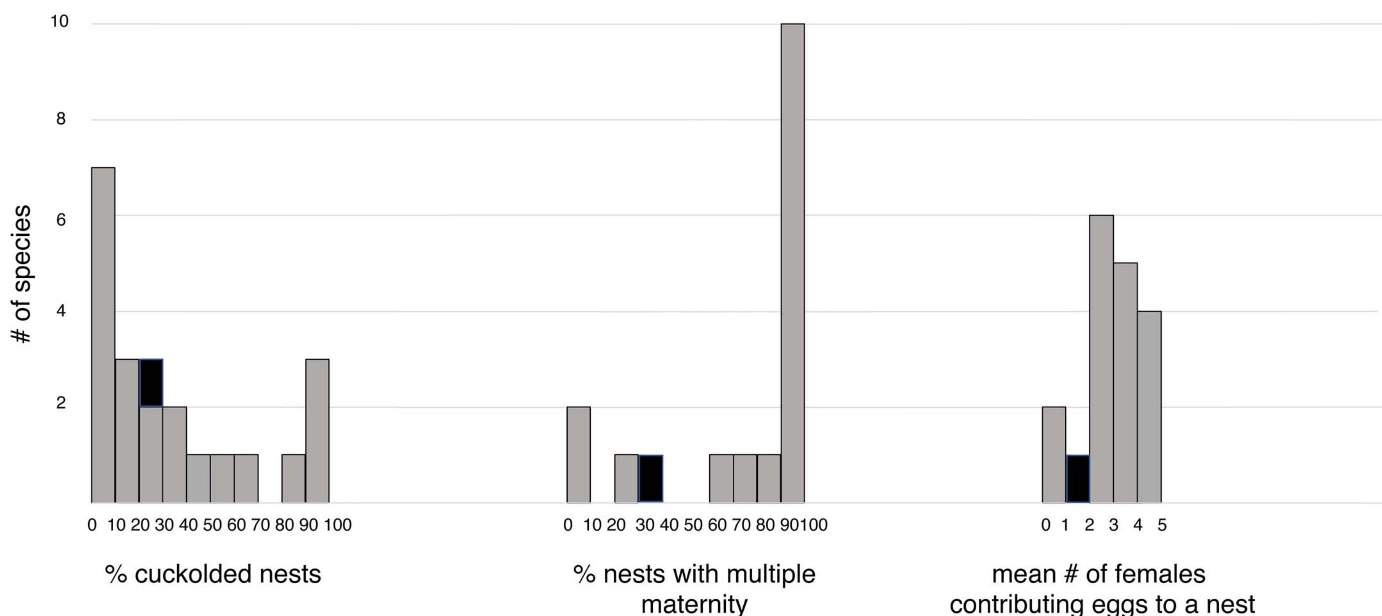

Fig. 3. Mating characteristics of bowfin relative to other nesting species with uniparental male care. Data represented by gray bars are from the review by Coleman and Jones (2011). Bowfin from this study are represented in black. Note that for bowfin relative to other species, the percentage of cuckolded nests is on the low side, that cases of multiple paternity are less common than for many species, and that the mean number of females spawning in a nest is relatively low.

bowfins compared to a phylogenetically broad range of nesting teleosts with uniparental male care (Fig. 3). In this context, we conclude that *Amia*, while behaviorally monogamous, is genetically only weakly polygynous. This result is particularly interesting because it has been suggested that promiscuous mating, where both males and females may have multiple partners, may be plesiomorphic for bony fishes (Turner, 1986).

Mating systems in non-teleost, non-tetrapod bony vertebrates: facts, speculations, and mysteries.—The diversity of mating strategies among non-teleostean actinopterygians and non-tetrapod bony vertebrates is both sufficiently great and sufficiently poorly known that it is not immediately clear whether promiscuity is, indeed, plesiomorphic for bony fishes as Turner (1986) suggested. Here, we review briefly what is known about spawning and mating system for lungfishes, coelacanths, and early diverging ray-finned fishes, including *Polypterus* (bichirs), sturgeon as represented by *Acipenser*, the American paddlefish, *Polyodon*, and gars, represented by *Lepisosteus* and *Atractosteus*.

Outside the Actinopterygii, both African and South American lungfishes are nest builders, with males providing parental care. For example, in *Protopterus annectens*, males excavate a u-shaped nest as much as 60 cm deep. The male has been observed attending "several females" during the breeding season (Bell-Cross and Minshull, 1988), but there are apparently no reports of additional males involved in spawning. Given this known natural history, polygyny or serial monogamy rather than promiscuity seem most likely for *Protopterus*. However, we know of no genetic study revealing the mating system of any lungfish.

The coelacanths, *Latimeria*, are viviparous with internal fertilization, and thus it seems unlikely that they are promiscuous. The rarity of coelacanths, along with the depth at which they live (Bruton and Stobbs, 1991) make it nearly impossible to observe spawning. However, a genetic assessment of the parentage of offspring could be done by sampling tissues from embryos within an individual female. As many as 26 embryos have been found in one female (Heemstra and Greenwood, 1992).

Among non-teleostean actinopterygians, little is known of reproduction in the African bichirs and their allies (Wiley, 1998) because spawning has not been observed in the wild. John Samuel Budgett, the British naturalist who mounted four remarkable expeditions to Africa to collect a growth series of *Polypterus* (Kerr, 1907a; Hall, 2001) succeeded in generating a growth series through artificial fertilization of eggs from wild-caught fish brought to him by local residents, but he never observed spawning. However, excerpts from Budgett's field notes were later published in the Budgett Memorial Volume (Kerr, 1907a), perhaps unnoticed, in Kerr's (1907b) description of the developmental series of *Polypterus senegalus* that Budgett collected. These field notes contain reports of floating nests of vegetation attributed to "Sayo," the name for bichirs used by local residents (Kerr, 1907b, p. 196–197). Of course, it is possible that these nests were constructed by fishes other than *Polypterus*. Because Budgett was unable to confirm these reports himself, he concluded that *Polypterus* "probably" does not build nests (Budgett, 1901, p. 123). In captivity, courtship and spawning behavior by male–female pairs have been described for bichirs (e.g., Budgett, 1907; Burgess, 1983), suggesting that they can be monogamous, but this does not necessarily mean that they are monogamous in nature. The fact that captive spawning requires providing the pair with thick vegetation or a spawning mop (Aquatic Community website, 2023. Breeding Bichirs. https://www.aquaticcommunity.com/predatory/breedingbichir.php) makes the mention of floating nests of vegetation in Budgett's field notes more intriguing. Given that spawning in the wild has not been observed, there are neither observational nor genetic data available pertinent to the nature of any polypterid mating system.

Spawning by *Polydon spathula*, the North American Paddlefish, has apparently only been observed in nature once (Jennings and Wilson, 1993). Over a gravel bar in the Osage River of Missouri, Purkett (1961) reported "the appearance of single fish which, while just visible at the surface, would agitate the caudal fin several times, then disappear after a few seconds. This occurred every few minutes throughout the late afternoon and evening." He interpreted this activity as spawning and surmised that "a spawning 'rush' occurred from a considerable distance under the surface during which the eggs were released by a rapid agitation of the caudal of the female." He continued, "Presumably, accompanying males then released milt. The surfacing appeared to be the end of the spawning rush of the female." However, there is no indication that he observed the accompanying male or males, and there are no genetic data indicating the number of parents involved in spawning. In the absence of observed spawning events, conservation and fisheries biologists generally infer successful reproduction from the presence of post-larval and juvenile paddlefish (Jennings and Wilson, 1993). Paddlefish are sometimes assumed to spawn like sturgeon (e.g., see Jarić et al., 2018) because they are closely related.

More is known about spawning in sturgeon. In *Acipenser fulvescens*, the Lake Sturgeon, between two and eight males spawn with a single female and a single male may then spawn with more than one female (Bruch and Binkowski, 2002). Polyandrous mating of multiple males with one female has also been described in the Shortnose Sturgeon, *A. brevirostrum*, with spawning events sometimes including the contributions of sneaker males (Kynard et al., 2016). Although spawning behavior has been difficult to observe in *A. transmontanus* (White Sturgeon) because they spawn in deep and turbid waters (Hildebrand et al., 2016), aerial surveys have documented one larger fish (putatively female) attended by two to three smaller putative males (unpublished data from Triton 2004 in Hildebrand et al., 2016). Genetic pedigree analysis, based on modeling a network of sibs and half-sibs represented in collections of eggs of *A. transmontanus*, estimates that 2.9 ± 2.5 adults are participating (Jay et al., 2014). Although the spawning behavior has not been observed for all sturgeon species, such as the Atlantic sturgeon, *A. oxyrinchus oxyrinchus* (Hilton et al., 2016), or for *A. oxyrinchus desotoi*, the Gulf Sturgeon (Sulak et al., 2016), there is strong observational evidence for polyandrous spawning by some species of sturgeon.

Spawning behavior of gars, the sister group to *Amia*, resembles what has been described for sturgeon more than spawning by *Amia*. Modern accounts of gar spawning behavior (e.g., Mendoza Alfaro et al., 2008; Smith et al., 2020), however, seem to rely on early observations of *Lepisosteus osseus*, the Longnose Gar, in Black Lake, New York (Dean, 1895). Males and females approach shallow water "already divided into [spawning] parties" from larger spawning aggregations, with each female attended by two to eight males (Dean, 1895). Dean continues

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that the fish come into very shallow water and then circle back farther from shore, with females depositing eggs and the males their milt. Males apparently do not "evidence rivalry," although Dean writes that when a male is unable to secure a place near the female he may swim "backward before the group, expanding his fins, while making side and upward motions with his head and paired fins." For the six other species of lepisosteids, we know more about spawning habitat and environmental drivers of spawning than we know about spawning behavior (Buckmeier et al., 2017), which is assumed to be similar to *L. osseus*. The Alligator Gar, *Atractosteus spatula*, spawns over wetland or terrestrial vegetation during flood pulses sufficient to inundate flood plains long enough for spawning and subsequent hatch of eggs before the water recedes (Buckmeier et al., 2017; Allen et al., 2020). In the only account of spawning by Alligator Gar, a larger female was observed to be attended by up to nine males, some of which seemed too small to be spawning (Kimmel et al., 2014). It would be interesting to discover the role of small males; are they sneakers or just young males going along with the "spawning party"? Also interesting is that the sex ratio of "spawning aggregations," before they divide into smaller "spawning parties," varies by species, and geographically within species. Sex ratios of spawning aggregations have been reported for different species as follows: for *L. platyrhincus* 1:1 male per female; for *L. osseus* 3:1 males per female; for *Atractosteus spatula* 2:1 and 1:1 males per female; for *A. tropicus* from 1:1, 3:1, to 5:1 males per female (García de León, 2001; Mendoza Alfaro et al., 2008). It would be interesting to know why these sex ratios are sometimes skewed and whether the sex ratio of aggregations affects sex ratios of spawning parties. To our knowledge, there has been no genetic assessment for any lepisosteid spawning of what proportion of males in a spawning party have successfully fertilized eggs for a given female.

In summary, we know surprisingly little about mating systems of non-teleostean actinopterygians. In part, this is likely due to the difficulty of observing spawning activity, especially when spawning occurs at night, in deep water, or during the tropical wet season. In the absence of observation, it has been assumed that promiscuous mating is plesiomorphic for teleost fishes, that there must be multiple males involved in spawning (even if they are not observed) as described above for bowfin and paddlefish, that paddlefish spawning must be the same as sturgeon, and that spawning behavior is consistent across species within a family (i.e., all sturgeons, all gars). Our review of the literature reveals that these assumptions might profitably be revisited because many mysteries remain about spawning behavior in non-teleost actinopterygians. We have learned for fishes and other organisms that mating systems, as evidenced by observation, are not necessarily the same as mating systems as evidenced by genetic analyses. The overall paucity of a genetic perspective on the mating systems of non-teleost actinopterygians brings new interest to genetic studies of these fascinating taxa.

DATA ACCESSIBILITY

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