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Multiple paternity in a natural population of a salamander with long-term sperm storage

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Abstract

Sperm competition appears to be an important aspect of any mating system in which female organisms mate multiply and store sperm. Postcopulatory sexual selection may be particularly important in species that store sperm throughout long breeding seasons, because the lengthy storage period may permit extensive interactions among rival sperm. Few studies have addressed the potential for sperm competition in species exhibiting prolonged sperm storage. We used microsatellite markers to examine offspring paternity in field-collected clutches of the Ocoee salamander (*Desmognathus ocoee*), a species in which female organisms store sperm for up to 9 months prior to fertilization. We found that 96% of clutches were sired by multiple males, but that the majority of females used sperm from only two or three males to fertilize their eggs. The high rate of multiple mating by females suggests that sperm competition is an important aspect of this mating system. Comparison of our data with those of other parentage studies in salamanders and newts reveals that multiple mating may be common in urodele amphibians. Nevertheless, the number of males siring offspring per clutch in *D. ocoee* did not differ appreciably from that in other species of urodeles with shorter storage periods.

Keywords: *Desmognathus ocoee*, microsatellite, parentage, salamander, sperm storage

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Introduction

Sperm competition and cryptic female choice have been extremely active areas of research in the last two decades, yet we still have much to learn about both processes. Although sperm competition and cryptic choice can occur in species with external fertilization, postcopulatory sexual selection seems to be most important in species that mate multiply and store sperm (Eberhard 1996; Birkhead & Møller 1998). Sperm storage has been documented in numerous animal taxa with internal fertilization, and most species store sperm for relatively short periods of time such as days or weeks (Birkhead & Møller 1993, 1998). Arguably, sperm competition and cryptic female choice may be expected to have greater potential importance in species that store sperm for very long periods of time (i.e. months or years; Birkhead & Møller 1993), because a wider array of mechanisms could come into play during periods of prolonged sperm storage than during very short storage intervals. Prolonged sperm storage (lasting more than 60 days) has been documented in several taxonomic groups, such as birds (Christensen 1981; Long *et al.* 2003), bats (reviewed by Racey 1975; Gomendio *et al.* 1998), fishes (Darling *et al.* 1980; Muñoz *et al.* 1999), reptiles (reviewed by Olsson & Madsen 1998; see also Adams & Cooper 1988; Schuett 1992; Aldridge & Duvall 2002; Sarkar *et al.* 2003), salamanders (reviewed by Halliday 1998; Sever 2002), and social insects (reviewed by Hölldobler & Wilson 1990) but, with the exception of social insects, relatively little work has been done on sperm competition and patterns of multiple mating in those species with prolonged sperm storage (but see Tilley & Hausman 1976; Labanick 1983; Hosken 1997; Mccracken *et al.* 1999; Pearse *et al.* 2001; Jones *et al.* 2002a, b).

One of the reasons that prolonged sperm storage could be important to the sexual selection process is simply that females storing sperm are able to accumulate sperm from more males before they fertilize their eggs, as might occur if females

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continue to mate during the storage period. Such a pattern of long-term sperm storage with mating during an extended breeding season has been documented in some snakes and turtles (reviewed by Olsson & Madsen 1998; see also Pearse *et al.* 2001), and salamanders (reviewed by Halliday 1998; Sever 2002). In species such as these, intense sperm competition might be expected, because fertilization of each egg could involve competing sperm from numerous males. This line of reasoning leads to the hypothesis that species with prolonged sperm storage and mating during the storage period will exhibit higher rates of concurrent multiple paternity (and consequently higher intensities of sperm competition) on average than species without long-term sperm storage.

The Ocoee salamander, *Desmognathus ocoee* (family Plethodontidae), offers an excellent opportunity to investigate the association between prolonged sperm storage during a long breeding season and high rates of concurrent multiple paternity. Found in the Appalachian Mountains of North Carolina, *D. ocoee* inhabits terrestrial sites, usually near running water (Tilley 1977). Male–female interactions in *D. ocoee* span a 9-month period from early fall to late spring (Organ 1961; Tilley 1977; Sever & Hamlett 1998) and conclude with the production of a single clutch of eggs by each reproductively active female. Sperm are stored in the female's spermatheca until ovulation, fertilization and oviposition, which occur in rapid sequence in June and July (Organ 1961). Stored sperm require hospitable physiological conditions to remain viable and may need female resources to survive until ovulation (Sever & Kloepfer 1993). However, sperm are degraded and cleared from the spermatheca several weeks after oviposition and sperm carry-over from one clutch to the next appears unlikely (Sever & Hamlett 1998; EMA, personal observation). Oviposition takes place on land, and the female tends her single clutch of 9–32 eggs for 6–8 weeks. Maternal care ends after hatching, and males are not known to provide resources to either the female or her offspring. Laboratory-based studies of mating behaviour in *D. ocoee* have shown that females may be inseminated up to 26 times by different males during a single breeding season (Houck *et al.* 1985a), although whether this high rate of insemination also occurs in the field is unknown. Apparent multiple paternity in nature has been documented in two studies of this species. These studies, based on allozymes and colour polymorphisms, suggested that at least 7–25% of females mate multiply in the field (Tilley & Hausman 1976; Labanick 1983), but the techniques employed lacked the resolution to provide anything better than a crude estimate. Clearly, the quantification of female mating patterns in this system requires the use of highly polymorphic markers, such as microsatellites.

Several studies have characterized mating patterns in amphibians, including a few which have found multiple paternity in anurans with external fertilization (D'Orgeix & Turner 1995; Roberts *et al.* 1999; Lodé & Lesbarrères 2003). A handful of studies have found multiple paternity in newts and salamanders (Gabor *et al.* 2000; Jones *et al.* 2002a, b, 2004; Rafinski & Osikowski 2002; Garner & Schmidt 2003; Tennessen & Zamudio 2003; Myers & Zamudio 2004). However, amphibian mating systems remain a largely untapped resource in the study of sexual selection and sperm competition, especially considering the variation among species with respect to mating behaviour, sperm transfer and sperm storage (Halliday 1998).

Our goal in the present study is to assess the potential for sperm competition in *D. ocoee* in nature. Specifically, we cloned and characterized novel microsatellite loci from this species and applied them to parentage analysis of field-collected clutches. This study allowed us to address three major questions. First, what is the rate of concurrent multiple paternity in this species with a high potential rate of multiple mating and long-term sperm storage? Second, is there evidence for unequal reproductive contributions among males within clutches? Third, how do the patterns of multiple mating in this species compare to those of newts and salamanders with shorter periods of sperm storage?

Methods

Microsatellite development

Microsatellite loci were identified using a modification of the enrichment protocol of Kijas *et al.* (1994), as described by Jones *et al.* (2001). In brief, a standard phenol–chloroform procedure was used to extract genomic DNA from a single *Desmognathus ocoee* individual collected at Deep Gap (Macon Co.), North Carolina (35°02′42″N, 083°33′19″W), and a partial genomic DNA library was constructed (Sambrook *et al.* 1989). A biotinylated oligonucleotide procedure was used to enrich the library for GATA-motif simple sequence repeats (Kijas *et al.* 1994; Jones *et al.* 2001). The enriched library was screened with a ^{32}P labelled $(GATA)_4$ oligonucleotide (Jones *et al.* 2001), and the inserts of positive recombinants were sequenced. Polymerase chain reaction (PCR) primer pairs were designed from the genomic DNA flanking each microsatellite repeat. Three primer pairs produced reliable, polymorphic results (Table 1).

Sample collection and genotyping

We collected 27 females with clutches plus one clutch whose female escaped (i.e. a total of 28 clutches) suitable for microsatellite analysis between 11 and 15 August 2001 from two localities (separated by less than 4 km) in the Nantahala Mountains of North Carolina. Twelve clutches were collected from Deep Gap and 16 were collected from Standing Indian (35°02′77″N, 083°30′63″W). Standing Indian and Deep Gap are connected by Kimsey Creek and likely represent parts of a single continuous population. Comparisons

Table 1 Variation in three novel *Desmognathus ocoee* microsatellite DNA loci in a sample of 42 individuals. Shown for each locus are the number and nature of tetranucleotide repeats in the original microsatellite clone, primer sequences, size range of alleles, number of unique alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), and optimal annealing temperatures for each locus

Locus	Repeat motif	Primer sequences (5′–3′)	Size range (bp)	No. of unique alleles	H_O	H_E	Optimal annealing temperature (°C)
Doc01	$(GATA)_8$ $(GATA)_3$	F: (6-FAM) TGTGAAGGGTGTTCTCTTACTG R: GCTGTTTGTGCTTTGACTTTAC	115–211	22	0.786*	0.905	52
Doc02	$(GATA)_{10}$	F: (HEX) TCAATCCAAGCACCATCAAAAG R: ACCCAAAACAGCACCAGCA	202–576	47	0.905*	0.976	56
Doc03	$(GATA)_{10}$	F: (NED) CTCTCCCACTCTTCCTCAAGTA R: CTTCACCTTCGCTATGACTGT	105–183	17	0.952	0.929	54

*denotes significant heterozygote deficit due to null alleles ($P < 0.01$).

of reproductive isolation between *D. ocoee* (formerly *Desmognathus ochrophaeus*) individuals from Standing Indian and Wayah Bald (approximately 15 km away) and John's Knob (more than 50 km away) revealed no differences between Standing Indian individuals and either of the other two sites (Tilley *et al*. 1990). Wayah Bald and John's Knob are both farther away from Standing Indian than is Deep Gap. In the present study, we found no evidence that our results differed between Standing Indian and Deep Gap.

Eggs and maternal tail-tip tissue samples were frozen on dry ice in the field and stored at −80 °C. Prior to molecular analysis, we dissected each egg to remove all surrounding membranes and yolk from the embryos. Genomic DNA was extracted from the embryonic tissue by using a standard proteinase K, phenol–chloroform procedure (Sambrook *et al*. 1989). All mothers and offspring were genotyped using the three tetranucleotide microsatellite loci. Each 20-μL PCR included a final concentration of 1 × *Taq* buffer, 1.5 mM $MgCl_2$, 0.5 μM of each forward and reverse primer, 0.1 mM of each dNTP, 0.5 U of *Taq* polymerase, and approximately 50 ng of template DNA. PCR conditions for each of the three loci differed from one another only in annealing temperature (52 °C–56 °C) and thermal cycling consisted of 5 min at 94 °C followed by 36 cycles of 1 min at 92 °C, 1 min at the annealing temperature, 2 min at 72 °C (Table 1). Thermal cycling concluded with 10 min at 72 °C. PCR fragments were resolved on an ABI 3100 Automated Sequencer and scored using GENESCAN fragment analysis software. We also assayed the microsatellite markers in an additional 42 unrelated adults collected at Deep Gap to calculate allele frequencies, observed and expected heterozygosities (Table 1), and to test for linkage disequilibrium and Hardy–Weinberg equilibrium at each of the three loci (GENEPOP version 3.1b, Raymond & Rousset 1995).

Parentage analysis

The analysis of maternal and offspring genotypes from field-collected samples was accomplished using the computer software GERUD version 1.0 (Jones 2001). For the one clutch whose mother escaped, only one heterozygous genotype was consistent with the entire progeny array at each locus, so we assumed that this heterozygous genotype was the maternal genotype. Thus, for all 28 clutches, we were able to determine the minimum number of males siring offspring in each clutch and to reconstruct the paternal genotypes consistent with the progeny array. In cases where more than one genotypic reconstruction was possible for the sires in a clutch, the program listed all possible paternal genotypic combinations and ranked the families based on probability criteria (Jones 2001). However, we chose conservatively to accept the program-generated paternal genotypic reconstructions only when one unique solution was obtained. Simulations run using GERUDSIM version 1.0 revealed that a unique minimum-father solution returned by GERUD 1.0 almost always represents the true paternal genotypes (see Jones 2001).

Reliability of parentage analysis

We used GERUDSIM version 1.0 simulation software to assess the probability of accurately detecting all sires in each clutch and to determine the probability of correctly reconstructing paternal genotypes from known maternal and offspring genotypes (Jones 2001). For each iteration of a given simulation, GERUDSIM 1.0 drew alleles at random from known allele frequencies at each locus, and then used those alleles to construct a single maternal genotype and several paternal genotypes. From the parental genotypes, GERUDSIM 1.0 constructed an offspring array based on a user-specified clutch size and paternity distribution. GERUDSIM 1.0 then removed the paternal genotypes from the analysis and, using the same analysis algorithm as in GERUD 1.0, attempted to determine the number of males siring offspring per clutch, as well as reconstructed all paternal genotypes (Jones 2001). Finally, these results were compared to the original paternal genotypes that were used to construct the simulated progeny array. We ran 1000 iterations to determine

the probability of correctly determining the number of males siring offspring per clutch and of correctly reconstructing all paternal genotypes for each hypothesized pattern of paternity.

Results

The *Desmognathus ocoee* tetranucleotide microsatellite markers were highly polymorphic with 17–47 alleles per locus, making them extremely informative for parentage analysis. No pairs of loci were found to be in linkage disequilibrium. One embryo was found to have a *de novo* mutation at the maternal allele (from 244 to 248 base pairs) at the *Doc02* locus, indicating that there had been a single-repeat mutation of the maternally inherited allele. However, mutations appeared not to be a major complication in this study. We found no evidence that *Doc03* deviated from Hardy–Weinberg equilibrium (HWE). However, each of the other two loci (*Doc01* and *Doc02*) displayed a significant heterozygote deficiency, almost certainly as a consequence of low frequency null alleles. The 42 individuals whose genotypes were included in the previous analyses were also used as parents in a laboratory parentage study (unpublished data) in which all their offspring were genotyped. Through the offspring arrays we were able to assess which parents had null alleles. In the current analysis, when we assigned individuals (= parents) having a null allele a numerical allele (for the null allele, i.e. '999'), both *Doc01* and *Doc02* returned to HWE.

Null alleles can be a major complication for parentage analysis. However, the estimated frequencies of null alleles at the loci used in this study are quite small (*Doc01*: 3.6%, *Doc02*: 4.7%, and *Doc03*: 1.2%). Furthermore, null alleles result in uncertainty regarding the number of sires per clutch only in clutches in which offspring appeared to be homozygous for one or the other of the heterozygous maternal alleles. When both maternal alleles appear in homozygous offspring, it is important to address the possibility that there really were not two unique paternal alleles, but a single paternal null allele. This situation occurred in 5 of our 28 clutches analysed (two clutches at the *Doc01* locus and three clutches at the *Doc02* locus). In all five of these cases, we conservatively assumed that the offspring shared a paternal null allele. Analysis with the other two loci (i.e. those without null alleles segregating in the progeny array) corroborated the interpretation that the apparently homozygous offspring were carrying a paternal null allele. Although null alleles have the potential to introduce uncertainty into any parentage analysis, they appear not to have affected our analysis because of their low frequencies in our samples.

Analysis of the 28 field-collected clutches revealed that only one clutch had been sired by a single male, 15 clutches had been sired by two males, nine clutches had been sired by three males, and three had been sired by four males (Fig. 1). Thus, most females (96%) produced clutches sired by more than one male. Of the multiply sired clutches, the vast majority (89%) were sired by either two or three males. A *t*-test comparing the mean number of fathers siring offspring per clutch between the two localities we sampled was not significant ($P > 0.10$).

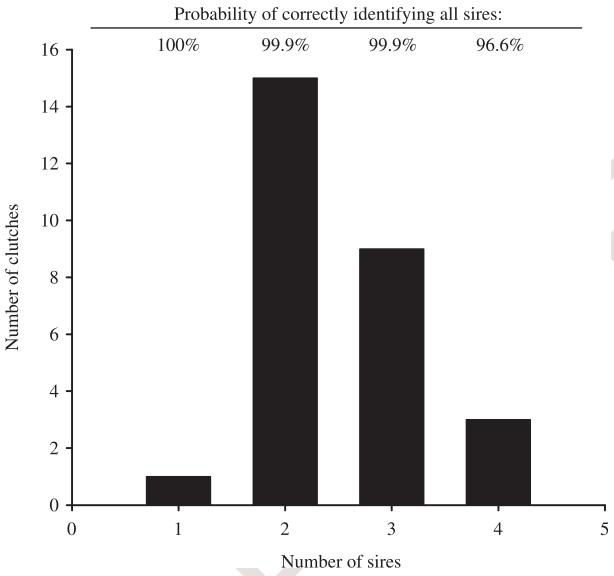

Fig. 1 Microsatellite parentage analysis of 28 field-collected clutches of *Desmognathus ocoee*. Female multiple mating is common in *D. ocoee* with 96% of the clutches having more than one sire, and 86% of clutches sired by either 2 or 3 males.

We were able to reconstruct unique paternal genotypic solutions for 13 of the 15 clutches sired by two males. In these 13 clutches, the most successful male sired between 58% and 86% of the offspring (Fig. 2a). Logistic regression analysis showed that these values deviated significantly from the null expectation of 50% (H_O = equal paternity among males, $P < 0.001$). For those clutches with either three or four sires, analysis with GERUD 1.0 resulted in a single unequivocal paternal genotype reconstruction for 6 of the 12 clutches. In each of these six clutches, the most successful male usually sired approximately half of the offspring (Fig. 2b).

We used the program GERUDSIM 1.0 to assess the reliability of GERUD 1.0 to determine the number of sires per clutch and to reconstruct paternal genotypes based on our microsatellite markers. Because our results from the GERUD 1.0 analysis determined that one male usually sired the majority of offspring in a clutch, we based our parameters in GERUDSIM 1.0 on the average clutch size and skewed distribution of paternity observed in the field data. Average clutch size was 15 eggs for broods with two sires, with 80% of the offspring sired by one male and 20% by the other, 19 eggs for broods with three sires (50%, 33% and 17% of offspring

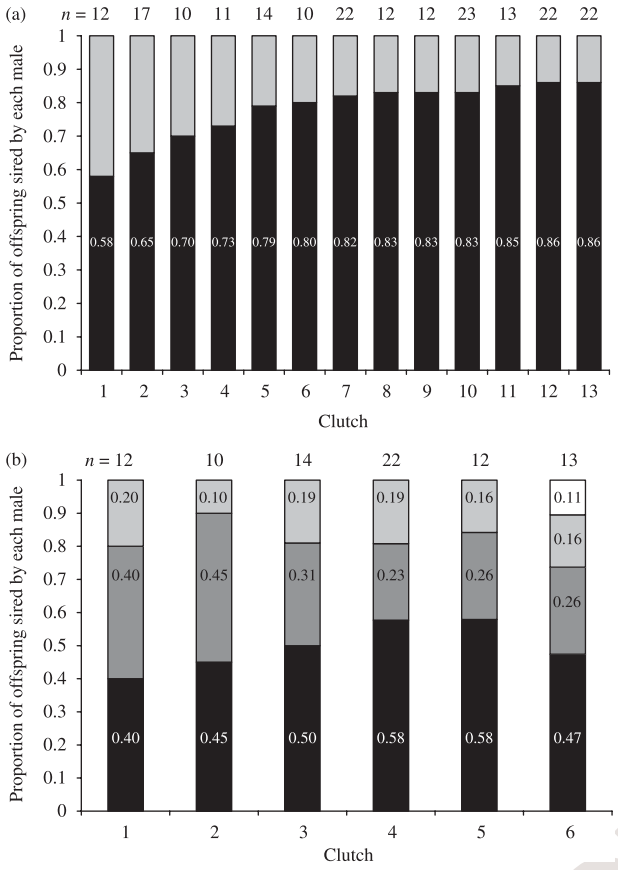

Fig. 2 (a) Distribution of paternity within clutches sired by two males. One male sires the majority of offspring in each clutch (H_O = equal paternity among males, $P < 0.001$). Numbers above each bar indicate the number of eggs in each clutch. (Note: bar colour does not imply a particular insemination order.) (b) Distribution of paternity within clutches sired by three and four males. One male tended to sire the majority of offspring in each clutch. Genotype reconstruction was possible for five of the clutches sired by three males, but only one of the clutches sired by four males. Numbers within the differently shaded regions of each bar reflect the respective proportion of offspring sired by each male. Numbers above each bar indicate clutch size. (Note: bar colour does not imply a particular insemination order.)

sired by each male, respectively), and 18 for those with four sires (47%, 26%, 16% and 11% of offspring sired by each male, respectively). The results of simulations examining the probability of correctly determining the number of males siring offspring per clutch ranged between 99.9% and 100% for clutches having between one and three sires, and 96.6% for clutches having four sires. Because the probability of correctly reconstructing all paternal genotypes represented in a clutch relies heavily on the number of offspring each male sired, we separated probabilities into two categories: one category for those males that sired six or more offspring and a second category for those males that sired fewer than six offspring. For males siring six or more offspring, there was a 96.6%, 88.0%, and 86.8% probability of correctly reconstructing their genotypes in clutches having two, three and four sires, respectively. Conversely, the probability of correctly reconstructing paternal genotypes for males siring fewer than six offspring in a clutch was 38.5%, 38.1%, and 33.0% in clutches having two, three and four sires, respectively.

Discussion

Our data indicate that a major requirement of sperm competition, multiple mating by females, is satisfied in this natural population of *Desmognathus ocoee*. Of the 28 clutches analysed, 96% are sired by more than one male. Thus, polyandry and resulting sperm competition may be important factors shaping the mating system of *D. ocoee*. High population densities in the field (Petranka *et al.* 1993), a long breeding season (Organ 1961; Tilley 1977; Sever & Hamlett 1998), and observations of high insemination rates in the laboratory (Houck *et al.* 1985a; up to 26 males in a single breeding season) suggested that female *D. ocoee* might mate frequently in the field and produce clutches sired by many males. While female multiple mating appears to be almost ubiquitous in the population we sampled, only a few males sire offspring in each clutch. Female insemination frequencies in the field are unknown, but during laboratory courtship encounters, females can and do reject males (Houck *et al.* 1985a, 1988; Verrell & Arnold 1989). Therefore, in a mating system with widespread multiple mating, these rejections suggest that females may be capable of controlling insemination and mating frequency.

Our data also reveal that one male tended to sire a majority of offspring in each clutch and that the proportion of offspring sired by the most successful male was relatively consistent across clutches with the same number of sires (Fig. 2a, b). This finding, together with evidence from Houck *et al.* (1985b), suggests that sperm precedence, potentially favouring the first male to inseminate a female, may be acting in *D. ocoee* (although we note that one of seven clutches in their study showed last-male sperm precedence). If so, sperm competition may have a large impact on male reproductive strategies, with males balancing the advantages in precedence from being the first to mate with a female against the disadvantage of having sperm in storage for a longer period of time.

The low number of sires per female observed in this study is not a consequence of methodological limitations. We used computer simulations to show that with our three microsatellite loci, we had a very high probability of correctly determining the number of sires per clutch, as well as correctly reconstructing most paternal genotypes. Still, GERUDSIM 1.0 clearly demonstrated the inherent problems associated with reconstructing paternal genotypes when males sire only a few offspring in a clutch. The primary

reason for the decline in our ability to correctly reconstruct paternal genotypes when more than two males sire offspring in a clutch is the small clutch sizes found in *D. ocoee*. The average size of *D. ocoee* clutches collected from the field was just 16 eggs (range of 9–32 eggs). As the number of males siring offspring per clutch increases the numbers of offspring per sire decreases. If several males sire fewer than six offspring each within a clutch, there is a substantial risk that not all of their alleles will segregate in the progeny array. However, if one male sires the majority of offspring in a clutch (or if a clutch is particularly large), the probability of correctly reconstructing that male's genotype is high, even if numerous males sire offspring in a clutch. The overall conclusions of the GERUDSIM 1.0 analysis are that we had very high power to determine correctly the number of sires contributing to a progeny array, and that we could accurately reconstruct paternal genotypes for those males siring six or more progeny. The genotypes of those males siring only a few offspring may not be absolutely correct, but as their exact genotypes do not enter into our analyses, this error is not a concern for the present study.

Our results expand on those of two previous parentage studies in this species. Tilley & Hausman (1976) used allozymes to assess paternity in over 150 field-collected clutches of *D. ochrophaeus* (now known as *D. ocoee*) and concluded that only approximately 7% had been sired by more than one male. In a separate study, Labanick (1983) used colour polymorphisms and assumptions of Mendelian inheritance to assess paternity in field-collected clutches of *D. ocoee*. He concluded that only two of eight clutches had been sired by multiple males. Both studies suffered from severe limitations imposed by the markers used to assess paternity, which most likely explains the disparity between our results. With the use of highly polymorphic microsatellites, we were able to assess paternity with a higher level of accuracy, and our results indicate that multiple mating in the field is far more common in natural populations of *D. ocoee* than was previously thought. While sexual selection acting prior to insemination has been studied extensively in *D. ocoee* (e.g. female choice and male mating success; Houck *et al*. 1985a, 1988; Verrell & Arnold 1989), our study is the first to demonstrate that polyandry and potentially sperm competition and cryptic female choice are large components of the mating system in the field.

Detailed studies of paternity have been carried out for two other species of urodele amphibians. Both of these studies also found evidence for multiple mating by females. For example, in field studies of the rough-skinned newt (*Taricha granulosa*), Jones *et al*. (2002a, b) found that over 60% of females used sperm from multiple males and that the polyandrous females used sperm from two to five males to fertilize their eggs. Myers & Zamudio (2004) recently found that from one to eight males sired offspring in clutches of the spotted salamander (*Ambystoma maculatum*). However, the estimates produced by Myers & Zamudio (2004) have very wide confidence limits and thus are difficult to compare with our results. Sperm storage occurs in *T. granulosa* and *A. maculatum*, but the storage periods are much shorter than those of *D. ocoee*. The rough-skinned newt stores sperm for several months and like *D. ocoee* (Sever & Hamlett 1998), appears not to store sperm between breeding seasons. *Ambystoma maculatum* stores sperm for only a matter of days (Petranka 1998). Thus, among the three species studied to date, an extended period of mating and female sperm storage does not appear to result in additional males siring offspring per clutch.

Overall, polyandry seems to be a common occurrence among caudate amphibians, and although *D. ocoee* stores sperm for very long periods and mates during the storage period, the patterns of paternity in this species are not appreciably different than those in the other species of newts and salamanders that have been studied. Given the interesting patterns of mating, sperm storage, and polyandry in newts and salamanders, it appears likely that additional research in amphibians will lead to informative general insights into mating strategies, sperm competition, and cryptic female choice.

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References

Adams CS, Cooper WE Jr (1988) Oviductal morphology and sperm storage in the keeled earless lizard *Holbrookia propinqua*. *Herpetologica*, **44**, 190–197.

Aldridge RD, Duvall D (2002) Evolution of the mating season in the pitvipers of North America. *Herpetological Monographs*, **16**, 1–25.

Birkhead TR, Møller AP (1993) Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society*, **50**, 295–311.

Birkhead TR, Møller AP (1998) *Sperm Competition and Sexual Selection*. Academic Press, London.

Christensen VL (1981) Effect of insemination intervals on oviductal sperm storage in turkeys. *Poultry Science*, **60**, 2150–2156.

D'Orgeix CA, Turner BJ (1995) Multiple paternity in the red-eyed treefrog *Agalychnis callidryas* (Cope). *Molecular Ecology*, **4**, 505–508.

Darling JDS, Noble ML, Shaw E (1980) Reproductive strategies in the surfperches. Multiple insemination in natural populations of the shiner perch *Cymatogaster aggregata*. *Evolution*, **34**, 271–277.

Eberhard WG (1996) *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press, Princeton, New Jersey.

Gabor CR, Krenz JD, Jaeger RG (2000) Female choice, male interference and sperm precedence in the red-spotted newt. *Behavioral Ecology*, **11**, 115–124.

Garner TWJ, Schmidt BR (2003) Relatedness, body size and paternity in the alpine newt, *Triturus alpestris*. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **270**, 619–624.

Gomendio M, Harcourt AH, Roldan ERS (1998) Sperm competition in mammals. In: *Sperm Competition and Sexual Selection* (eds Birkhead TR, Møller AP), pp. 667–751. Academic Press, London.

Halliday TR (1998) Sperm competition in amphibians. In: *Sperm Competition and Sexual Selection* (eds Birkhead TR, Møller AP), pp. 465–502. Academic Press, London.

Hölldobler B, Wilson EO (1990) *The Ants*. Springer-Verlag, Berlin.

Hosken DJ (1997) Sperm competition in bats. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **264**, 385–392.

Houck LD, Arnold SJ, Hickman AR (1988) Tests for sexual isolation in plethodontid salamanders (genus *Desmognathus*). *Journal of Herpetology*, **22**, 186–191.

4 Houck LD, Arnold SJ, Thisted RA (1985a) A statistical study of mate choice: sexual selection in a plethodontid salamander (*Desmognathus ochrophaeus*). *Evolution*, **39**, 370–386.

Houck LD, Tilley SG, Arnold SJ (1985b) Sperm competition in a plethodontid salamander: preliminary results. *Journal of Herpetology*, **19**, 420–423.

Jones AG (2001) GERUD 1.0: a computer program for the reconstruction of parental genotypes from progeny arrays using multilocus DNA data. *Molecular Ecology Notes*, **1**, 215–218.

Jones AG, Adams EA, Arnold SJ (2002a) Topping off: a mechanism of first-male sperm precedence in a vertebrate. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 2078–2081.

Jones AG, Arguello JR, Arnold SJ (2002b) Validation of Bateman's principles: a genetic study of mating patterns and sexual selection in newts. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **269**, 2533–2539.

Jones AG, Arguello JR, Arnold SJ (2004) Molecular parentage analysis in experimental newt populations: the response of mating system measures to variation in the operational sex ratio. *American Naturalist*, **164**, 444–456.

Jones AG, Blouin MS, Arnold SJ (2001) Genetic variation in two populations of the rough-skinned newt (*Taricha granulosa*) assessed using novel tetranucleotide microsatellite loci. *Molecular Ecology Notes*, **1**, 293–296.

Kijas JMH, Fowler JCS, Garbett CA, Thomas MR (1994) Enrichment of microsatellites from the *Citrus* genome using biotinylated oligonulectide sequences bound to streptavidin coated magnetic particles. *BioTechniques*, **16**, 657–662.

Labanick G (1983) Inheritance of the red-leg and red-cheek traits in the salamander *Desmognathus ochrophaeus*. *Herpetologica*, **39**, 114–120.

Lodé T, Lesbarrères D (2003) Multiple paternity in *Rana dalmatina*, a monogamous territorial breeding anuran. *Naturwissenschaften*, **91**, 44–47.

Long EL, Sonstegard TS, Long JA, Van Tassel CP, Zuelke KA (2003) Serial analysis of gene expression (Sage) in turkey sperm storage tubules in the presence and absence of sperm. *Biology of Reproduction*, **69**, 469–474.

Mccracken GF, Burghardt GM, Houts SE (1999) Microsatellite markers and multiple paternity in the garter snake *Thamnophis sirtalis*. *Molecular Ecology*, **8**, 1475–1479.

Muñoz M, Casadevall M, Bonet S (1999) Annual reproductive cycle of *Helicolenus dactylopterus dactyloperus* (Teleostei: Scorpaeniformes) with special reference to the ovaries sperm storage. *Journal of the Marine Biological Association of the United Kingdom*, **79**, 521–529.

Myers EM, Zamudio KR (2004) Multiple paternity in an aggregate breeding amphibian: the effect of reproductive skew on estimates of male reproductive success. *Molecular Ecology*, **13**, 1951–1963.

Olsson M, Madsen T (1998) Sexual selection and sperm competition in reptiles. In: *Sperm Competition and Sexual Selection* (eds Birkhead TR, Møller AP), pp. 503–577. Academic Press, London.

Organ JA (1961) Studies on the local distribution, life history, and population dynamics of the salamander genus *Desmognathus* in Virginia. *Ecological Monographs*, **31**, 189–220.

Pearse DE, Janzen FJ, Avise JC (2001) Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. *Heredity*, **86**, 378–384.

Petranka J (1998) *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, D.C.

Petranka JW, Eldridge ME, Haley KE (1993) Effects of timber harvesting on southern Appalachian salamanders. *Conservation Biology*, **7**, 363–370.

Racey PA (1975) The prolonged survival of spermatozoa in bats. In: *The Biology of the Male Gamete* (eds Duckett JG, Racey PA), pp. 385–416. Academic Press, London.

Rafinski J, Osikowski A (2002) Sperm mixing in the alpine newt (*Triturus alpestris*). *Canadian Journal of Zoology*, **80**, 1293–1298.

Raymond M, Roussett F (1995) GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Roberts JD, Standish RJ, Byrne PG, Doughty P (1999) Synchronous polyandry and multiple paternity in the frog *Crinia georgiana* (Anura: Myobatrachidae). *Animal Behaviour*, **57**, 721–726.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sarkar S, Sarkar NK, Maiti BR (2003) Oviductal sperm storage structure and their changes during the seasonal (dissociated) reproductive cycle in the soft-shelled turtle, *Lissemys punctata punctata*. *Journal of Experimental Zoology*, **295A**, 83–91.

Schuett GW (1992) Is long-term sperm storage an important component of the reproductive biology of temperate pitvipers? In: *Biology of the Pitvipers* (eds Campbell JA, Brodie ED Jr), pp. 169–184, Selva, Tyler, Texas.

Sever DM (2002) Female sperm storage in amphibians. *Journal of Experimental Zoology*, **292**, 165–179.

Sever DM, Hamlett WC (1998) Sperm aggregations in the spermatheca of female desmognathine salamanders (Amphibia: Urodela: Plethodontidae). *Journal of Morphology*, **238**, 143–155.

Sever DM, Kloepfer NM (1993) Spermathecal cytology of *Ambystoma opacum* (Amphibia: Ambystomatidae) and the phylogeny of sperm storage organs in female salamanders. *Journal of Morphology*, **217**, 115–127.

Tennessen JA, Zamudio KR (2003) Early-male advantage, multiple

paternity and sperm storage in an amphibian aggregate breeder. *Molecular Ecology*, **12**, 1567–1576.
Tilley SG (1977) Studies of the life histories and reproduction in North American plethodontid salamanders. In: *The Reproductive Biology of Amphibians* (eds Taylor DH, Guttman SI), pp. 1–42. Plenum Press, New York.
Tilley SG, Hausman JS (1976) Allozymic variation and occurrence of multiple insemination in populations of the salamander *Desmognathus ochrophaeus*. *Copeia*, **1976**, 734–741.
Tilley SG, Verrell PA, Arnold SJ (1990) Correspondence between sexual isolation and allozyme differentiation: a test in the salamander *Desmognathus ochrophaeus*. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2715–2719.
Verrell PA, Arnold SJ (1989) Behavioral observations of sexual isolation among allopatric populations of the mountain dusky salamander, *Desmognathus ochrophaeus*. *Evolution*, **43**, 745–755.

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