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Multiple paternity in *Rana dalmatina*, a monogamous territorial breeding anuran

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Abstract Polyandry and sperm competition in anurans have rarely been documented. We investigated the genetic paternity inferred from allozyme variations in 650 tadpoles from four natural ponds in a territorial breeding anuran, *Rana dalmatina*. Multiple paternity was demonstrated, although *R. dalmatina* is regarded as a monogamous species. Polyandrous mating was not a common event, occurring only in 17.9% of clutches, with no significant differences among clutches. The proportions of tadpoles fathered by a second male did not significantly differ among ponds, showing that multipaternity was not restricted to a single site. Such a polyandry may result from synchronous multiple amplexus and should reduce the heterozygote deficit related to the breeding-pond fidelity usually exhibited by most anurans.

Introduction

Recent empirical studies have revealed a growing body of evidence for multiple matings in numerous species formerly thought to be socially monoandrous. Facilitated by sperm storage, polyandry is ubiquitous in insects (Arnqvist and Nilsson 2000) and extra-pair paternity has also been inferred in reptiles (Madsen et al. 1992), birds (Birkhead and Møller 1995; Hasselquist et al. 1996; Questiau et al. 1999; Double and Cockburn 2000) and mammals (Hoogland 1998; Schenk and Kovacs 1995; Wilmer et al. 2000). Many features of frog life histories suggest that multiple mating and sperm competition should be common phenomena in anurans (Roberts et al. 1999). For example, they have external fertilisation and weak or inconsistent paternal care, and the promiscuity of males chorusing in breeding congregations (Halliday and Tejedo 1995) should facilitate multiple paternity in egg

masses by simple spermatid diffusion. Moreover, many anuran species exhibit both a noticeable sexual size dimorphism in favour of females and a male-biased sex ratio (Geisselmann et al. 1971; Blab 1986; Reading et al. 1991), since males arrived precociously and usually stayed for a longer time than females in the breeding pond. Such a male-biased sex ratio could increase competitive interactions and may result in multiple males amplexing with a single female (Fukuyama 1991; Jennions et al. 1992; Halliday and Tejedo 1995).

Although multiple amplexus has been commonly observed in numerous anuran species, only three studies have referred to genetic polyandry within a single clutch in nature. Multiple amplexus resulted in multiple paternity in the red-eyed tree-frog, *Agalychnys callidryas*, as revealed by DNA fingerprinting (D'Orgeix and Turner 1995), and synchronous polyandry was demonstrated by allozyme electrophoresis in *Crinia georgiana* (Roberts et al. 1999). Finally, the high multiple paternity inferred from genetic analysis of egg masses in *Rana temporaria* (Laurila and Seppä 1998) is likely to be related to the promiscuous breeding behaviour of the species, which would lead to multiple paternity by diffuse spermatid fertilisation.

Several anuran species were rarely or never observed to have multiple amplexus, so that they could be considered as socially monoandrous species. Males may have several successive amplexus, but one female releases a single clutch during the breeding season. Furthermore, agile frogs do not form choruses and, like most precocious breeding anurans, they do not forage during the breeding season, suggesting that neither resource dispersion nor the need for parental care clearly influences their reproductive behaviour.

The low rate of multiple amplexus observed in anurans may indicate that the costs involved in multiple mating are higher than the advantages. Nevertheless, mating with several males may be advantageous for females by potentially increasing fertilisation, sperm quality or offspring genetic diversity, so that multiple matings may be more common than reported (Roberts et al. 1999).

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Although the life history, reproductive characteristics, and published observations (Geisselmann et al. 1971; Blab 1986) would suggest that *R. dalmatina* is a socially monoandrous species here, we report evidence of multiple paternity in a single clutch in wild populations of *R. dalmatina*.

Methods

Species reproduction

The agile frog, *Rana dalmatina*, is a widely distributed species found throughout Europe. This terrestrial and nocturnal anuran breeds in small breeding congregations from February to March. Males arrive some days earlier than females and spend more time in the pond. Frogs amplexing are distant from each other and it mainly occurs at night. Each female lays one clutch per breeding season and each male occupies a separate territory so that we were able to collect eggs from different amplexing pairs to test paternity.

Sample collection

Clutches were sampled in four distant ponds near Redon (47°34'N, 2°50'W), western France from 1998 to 2000. Less than 10% of eggs were randomly collected from 28 separated clutches to avoid diffuse fertilisation. Hatching tadpoles ($n=22-24$ per clutch for a total of 650 tadpoles) were reared for 20 days in constant environmental conditions in the laboratory. In regard to ethical considerations, only 22–24 tadpoles from collected clutches were reared in separate pans and then instantaneously killed for genetic analysis using MS222, while the others were released on the site.

Estimation of multiple paternity

Following Laurila and Seppä (1998) and Roberts et al. (1999), paternity was inferred from allozymic data by starch gel electrophoresis using standard techniques. Samples were homogenised in an equal volume of distilled water and centrifuged at 10,000 *g* for 15 min at 4°C. Electrophoretic migration was carried out using two

Table 1 Polymorphic loci successfully resolved in *Rana dalmatina* tadpoles from four ponds as revealed by starch-gel electrophoresis

Enzyme systems	EC number	Loci	Number of alleles
α -GDH	1.4.3.1	α - <i>gdh</i>	3
LDH	1.1.1.27	<i>Ldh-1</i>	3
	1.1.1.27	<i>Ldh-2</i>	5
MPI	5.3.1.8	<i>Mpi</i>	4
PGM	2.7.5.1	<i>Pgm</i>	2
6-PGDH	1.1.1.44	<i>6-Pgdh</i>	3
			Mean = 1.33 SD 1.03

Table 2 Allelic frequencies, observed and expected heterozygosities in 28 clutches of *Rana dalmatina* (the most common allele was designated a)

Locus	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>H</i> Obs	<i>H</i> Exp	<i>n</i>
α - <i>gdh</i>	0.5846	0.3015	0.1138			0.3462	0.5548	650
<i>Ldh-1</i>	0.4423	0.3485	0.2092			0.5338	0.6397	650
<i>Ldh-2</i>	0.5110	0.2136	0.1588	0.0750	0.0391	0.3991	0.6610	639
<i>Mpi</i>	0.5809	0.2465	0.1279	0.0447		0.4052	0.5839	649
<i>Pgm</i>	0.6277	0.3723				0.2738	0.4677	650
<i>6-Pgdh</i>	0.4035	0.4545	0.142			0.4892	0.6109	648
						0.4079	0.5863	
						SD 0.0942	SD 0.0694	

buffer systems, Tris-citrate pH6 and Tris-EDTA-borate pH8, and slices were stained for five specific enzymes encoded by six polymorphic loci with 2–5 alleles (Table 1). From allozymic data, *F*-statistics were derived using Genetix software (Belkhir, <http://www.Genetix@crit.univ-montp2.fr>). Allozyme phenotypes were evidence for heritable genotypes, and polyandry was estimated using PAPA 1.0 (Duchesne et al. 2002). The purpose was to determine a minimal set of loci based on the expected number of parents, the possibility of sexing parents, and the level of genotyping error. For diploid animals, the parentage allocation method used in PAPA is based on the likelihood that a parental pair produces multilocus genotypes found in the tested offspring. All potential parents are expected to show equal reproductive capability and mating is assumed to be potentially random. The validity of the allocation process is not affected by some departure from the latter conditions. Deviation from single mating for each clutch was determined by a simulation of parental genotypes, thus allowing us to assess the minimal number of parents for each clutch. Since every female produced a single clutch during the breeding season, monoandry refers to genetic evidence of mating with a single male and polyandry with at least two males.

Results

As inferred from multiple allelic allozymes, five loci (α -*gdh*, *Ldh-1*, *Ldh-2*, *Mpi* and *6-Pgdh*) showed at least three alleles, but enzyme *Pgm* had a di-allelic pattern (Table 1). The effective number of alleles per locus was 3.33. Observed heterozygosity averaged $H_O=0.408$ over loci (SD 0.0942, Table 2). Locus *Ldh-1* showed the highest heterozygosity level, whereas *Ldh-2* with five alleles exhibited the lowest heterozygosity level.

Most clutches (i.e. 82.1%, $n=23$ of 28 clutches) revealed a single paternity per clutch using different simulations. However, for five clutches (17.9%), it was unlikely that only one male had fathered all the offspring, providing evidence of multipaternity. Multiple paternity was detected in five clutches from three ponds.

However, in each multi-paternal clutch, a single male fertilised on average 76.2% of eggs while only 23.8% of eggs could be attributed to a second male (Table 3). Proportions of tadpoles fathered by a second male did not significantly differ among clutches ($z=-1.207$, $P=0.114$) and among ponds ($z=-0.444$, $P=0.33$). Because widely separated clutches were sampled from different male territories, these results could not be attributable to two females.

Table 3 Offspring proportion resulting from a fertilisation by a second male in five multiple paternal clutches as inferred from PAPA software

		<i>n</i> analysed offspring	<i>n</i> estimated fathers	Proportion of offspring corresponding to the first male	Proportion of offspring corresponding to a second male
Pond 1	Clutch 1	24	2	0.708	0.291
	Clutch 2	24	2	0.792	0.208
Total pond 1		48		0.750	0.250
Pond 3	Clutch 3	22	2	0.727	0.273
	Clutch 4	22	2	0.818	0.182
Total pond 3		44		0.773	0.227
Pond 4	Clutch 5	22	2	0.773	0.227
Total pond 4		22	2	0.773	0.227
Total		114	Mean = 2	Mean = 0.763	Mean = 0.238

Discussion

Although *Rana dalmatina* mainly shows characteristics of a monoandrous mating system, multiple paternity was demonstrated in this analysis. Multiple paternity occurred in three ponds, revealing that polyandry was not restricted to a single site.

Other polyandrous species have typically high levels of multiple paternity. For example, in *Rana temporaria* it occurred in half of the eggs (Laurila and Seppä 1998) and in *Crinia georgiana* half of all matings were polyandrous (Roberts et al. 1999). However, in *Rana dalmatina*, multiple paternity in a clutch from a single female is not a common event, and most of the clutches were only fathered by a single male. In polyandrous clutches, the second male only partially fertilised the clutch. Nevertheless, the number of males fathering offspring may be underestimated in agile frogs both because allozymic variations were not the best genetic marker (low level of polymorphism compared with microsatellites) for polyandry and because of the breeding-site fidelity of most anurans (see Reading et al. 1991). Breeding site fidelity should result in increasing relatedness of breeding adults.

Three main hypotheses could explain the proximate cause of polyandry in *Rana dalmatina*. Firstly, multiple paternity could result from spermatic diffusion from the sperm of neighbouring males fertilising eggs. Such a phenomenon would only be possible if spermatozooids could survive for several minutes when exposed to water. Furthermore, clutches would need to be in close proximity in order to facilitate fertilisation. As an example, a large breeding group of *Rana temporaria* was suggested as a mechanism to allow spermatic diffusion, resulting in multiple paternity (Laurila and Seppä 1998). By contrast, in *Rana dalmatina*, amplexing pairs and clutches are distant from each other, reducing the likelihood of diffuse fertilisation.

A second hypothesis is that polyandry may result from a secondary fertilisation. Jennions and Passmore (1993) demonstrated the possibility of sperm release by a subsequent second male in *Chiromantis*. Our data did not allow us to reject this hypothesis, although in *Rana dalmatina* a secondary fertilisation is unlikely because

male territorial behaviour persists for some days after amplexus, preventing other males from approaching the clutch.

A third possibility is that multiple paternity may result from synchronous mating. Competitive interactions do not prevent multiple amplexus in numerous anurans, and polyandry should be influenced by a male-biased sex ratio. Multiple paternity arises from multiple amplexus both in *Agalychnis* and *Crinia* (D'Orgeix and Turner 1995; Roberts et al 1999). In *Crinia* (Roberts et al 1999), the second amplexus was rarely dorsal, but rather the second male amplexed laterally or even ventrally. Such amplexus have been assumed to affect fertilisation success. The breeding season in *Rana dalmatina* is brief, restricted to about 10 days in February and March (Lesbarrères and Lodé 2002), and we observed some multiple amplexus during this time period, thus supporting the synchronous polyandry hypothesis.

Although females should be able to maximise their fitness by mating with a single partner, polyandry may confer genetic advantages (Yasui 1998; Hosken and Blanckenhorn 1999). Because of their pond fidelity (see Reading et al. 1991), anurans are affected by heterozygote deficit (Lesbarrères et al. 2003) and it may be suggested that polyandrous mating results in inbreeding avoidance.

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