



# Synchronous polyandry and multiple paternity in the frog *Crinia georgiana* (Anura: Myobatrachidae)

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Multiple paternity has rarely been reported in anuran amphibians, with only three previous documented examples. For the Australian frog *Crinia georgiana*, we observed synchronous polyandry in an average of 44% of matings observed at four field sites. This suggests matings involving more than one male are common in this species. One to eight males were observed in amplexant groups with second males amplexed ventrally. Genetic analyses, using allozyme electrophoresis, of offspring from two matings indicated that at least two of three possible males fathered offspring. Third males were unlikely to have shared paternity, explained by their inappropriate position during amplexus. Multiple paternity may be more common in frogs than has been reported.

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Multiple paternity and sperm competition are common phenomena in animal groups with both internal and external fertilization (e.g. Parker 1970; Smith 1984; Birkhead & Møller 1992, 1998). In anuran amphibians, where there is generally external fertilization (e.g. Halliday & Tejedo 1995), reports of multiple males in amplexus with, or associated with, single females are common (e.g. Roberts 1994; Halliday & Tejedo 1995; Halliday 1998). In many cases these reports raise the possibility of simultaneous sperm release by two or more males leading to sperm competition and multiple paternity of single-egg clutches (e.g. Feng & Narins 1991; Fukuyama 1991; Jennions et al. 1992; Kaminsky 1997). However, only one paper reports direct, genetic evidence of multiple paternity in anurans. Using DNA fingerprint analysis, D'Orgeix & Turner (1995) showed that both males amplexed with ovipositing females of the phylomedusid frog *Agalychnis callidryas* fertilized eggs with about equal frequency. Jennions & Passmore (1993) demonstrated sperm release by more than one male but not multiple paternity. When focal males were precluded from releasing sperm on to eggs in the rhacophorid frog *Chiromantis xerampelina*, peripheral males in the same foam nest fertilized ca. 40% of eggs released (Jennions &

Passmore 1993). Laurila & Seppa (1998) also inferred a high frequency of multiple paternity in egg masses of *Rana temporaria* in southern Finland. Their analysis was based on indirect genetic measures and could be in error if assumptions that egg masses were the product of single females, or relatedness amongst males was greater than zero, proved incorrect (Laurila & Seppa 1998).

For females, mating with multiple males may have several advantages. For example, females may avoid any risk of low sperm number, low sperm quality or problems of incompatibility with sperm from a single male or low genetic variance among offspring (e.g. Feng & Narins 1991; Bourne 1993; Olsson et al. 1996; Zeh 1997; Laurila & Seppa 1998). There may also, however, be a cost to mating with two or more males (summarized in Stockley 1998) resulting particularly from male tactics aimed at reducing the risk of sperm competition. The apparently low frequency of multiple paternity in anurans may indicate that these advantages do not exist for frogs or that they are outweighed by major costs. However, it is equally probable that the frequency of multiple paternity has simply been underestimated.

In this paper we report multiple males mating with single females in the Australian myobatrachid frog *Crinia georgiana*. Similar reports for other frogs have suggested, but generally not tested, that multiple paternity results from polyandrous matings. Therefore we also report an analysis of paternity in a limited sample of egg masses to determine whether multiple paternity is a possible outcome.

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**Table 1.** Frequency of matings with one or more males at four sites

Site	Number of males per mating								% Multiple	N	
	1	2	3	4	5	6	7	8			
Boulder Rock	1	1								50.0	2
Tash's Rock	8	3	2							38.5	13
Kangaroo Gully	42	17	13	7	5	0	2	2		52.2	88
Margaret River	2	1								33.3	3

## METHODS

### Mating Behaviour

*Crinia georgiana* is a small frog found throughout south-western Australia (Tyler et al. 1994). At our main study site, Kangaroo Gully, females (all measured after egg deposition) weighed  $1.8 \pm 0.099$  g ( $\bar{X} \pm$  SE, range 0.7–3.8,  $N=71$ ) and males weighed  $2.39 \pm 0.065$  g (range 0.8–6.4,  $N=249$ ). Egg deposition results in about a 33% drop in body mass for females ( $N=11$ ) so, prior to mating, average body masses are similar for both sexes. *Crinia georgiana* breeds in winter from June to September (Main 1965). Amplexus in this species is normally inguinal, as in most myobatrachid frogs (Littlejohn et al. 1993), and eggs are deposited in discrete clumps in shallow water (Seymour & Roberts 1995).

We studied mating behaviour of this species at four locations: Boulder Rock (10 August 1992), Tash's Rock (22 July–9 September 1996, 2–3 nights per week) and Kangaroo Gully (12 June–9 September 1997, 3–4 nights per week), all granite outcrops with shallow water and moss beds (cf. Seymour & Roberts 1995), 35–40 km south-east of Perth, Western Australia and at Margaret River (12 August 1997), 230 km southsouthwest of Perth in an area of flooded grass adjacent to a creek.

We located amplexing frogs by active searching through choruses or by making repeated observation of single females until they mated. It is possible that there is some bias towards detecting polyandrous matings as these may be more obvious than monandrous matings. The study sites were open with shallow water (1–2 cm deep) over granite or moss beds and frogs were obvious so we do not suspect any major bias. We recorded the number of males in amplexus with females and for a subset of matings recorded male position. In all cases amplexed frogs were depositing eggs.

### Paternity Analysis

We collected all eggs deposited from the only two three-male matings observed at Tash's Rock. We used matings with three potential fathers to maximize the possible levels of paternity. Matings with more males were likely to be beyond the resolution of our analytical techniques (see results for third males). Hatchling tadpoles were reared to metamorphosis. Whole frogs or toes were stored at  $-80^\circ\text{C}$  before electrophoresis (see Ethical Note below).

Multiple locus genotypes of adults and offspring were analysed by gel electrophoresis using standard techniques (Richardson et al. 1986). We extracted material for electrophoresis from toes of adults, from whole limbs of offspring, or from liver. Of 18 enzyme systems scored, two, glucose-phosphate isomerase (*Gpi*, EC 5.3.1.9) and leucyl-glycyl-glycine peptidase (*Lggp-2*, EC 3.4.1.3), showed variation that could be used as paternity markers.

Tissues were ground in an equal volume of buffer solution (Tris-EDTA-borate, pH 8, containing 0.1% mercaptoethanol and 0.02% bromophenol blue) and loaded on to horizontal starch gels (11.3% w/v Starch-Art) for electrophoresis. To facilitate scoring, groups of four offspring were run between putative parents. Alleles were labelled alphabetically in order of decreasing mobility.

We considered two issues: (1) whether there was any evidence of multiple paternity and, if so, (2) whether paternity was shared equally amongst all possible fathers.

For both matings (denoted A and B below) we scored the occurrence of genotypes specific to males 1, 2 or 3. We also analysed paternity using models based on one, two or three males sharing paternity equally, assuming codominant expression and Mendelian inheritance of alternate alleles. Alternative models of shared paternity could be fitted to these data and may show an equally good or possibly better fit. However, there is no a priori way to predict any particular sharing pattern and we have therefore assumed equality as a first, null model.

### Ethical Note

Adult frogs from mating A and metamorphlings were killed instantaneously and humanely by pithing. Adults were collected in mating A to give the best chance of finding enough electrophoretic variation to assign paternity with certainty. Single toes were clipped from adults in mating B and the adults released to minimize the impact on the adult frog population. Toe clipping does not appear to have any major impact on frog survival for species not living a fully aquatic existence and is preferable to killing the frog (Halliday 1995; van Gelder & Strijbosch 1996; cf. Golay & Durrer 1994). We collected only two egg clutches, as our goal was to demonstrate the possibility of multiple paternity rather than to give a detailed analysis of paternity patterns. Such analysis will require more sensitive markers (e.g. microsatellites).

(a)



(b)



**Figure 1.** (a) Photograph, taken in the field on 22 July 1996, illustrating amplexus position for males during egg release in the mating reported in Table 3. (b) Diagrammatic interpretation of photograph in (a). Individual frogs are identified by shading patterns: female: white; male 1: diagonal shading; male 2: black; male 3: spots. Eggs are black, white, or black and white circles.

## Mating A

Marker	Mother	Possible fathers			Possible offspring		
		1	2	3	1	2	3
<i>Gpi</i>	a						
	b						
<i>Lgsp-2</i>	a						
	b						
	c						

## Mating B

Marker	Mother	Possible fathers			Possible offspring		
		1	2	3	1	2	3
<i>Gpi</i>	a						
	b						
	a						

**Figure 2.** Assignment of paternity for matings A and B. For both matings we illustrate genotypes for the mother, potential fathers and possible offspring for all fathers. Observed offspring types are given in Tables 2 and 3. In mating A, genotypes are from two loci: both panels must be examined simultaneously as either genotype for *Gpi* can be combined with either genotype for *Lgsp-2*. Both loci were dimeric so heterozygotes exhibit both alleles plus an intermediate band indicated by a dashed line (e.g. mating A, Mother, *Gpi*: heterozygous *ab*; mating B, father 2, *Gpi*: heterozygous *ac*). Homozygotes are indicated by a broader black line. Mating A: dashed arrow indicates the *bb* genotype for *Gpi* expected if male 3 had a paternity share. No offspring of this genotype were observed (Table 2). If male 3 is therefore excluded as a father, black arrows for *Lgsp-2* indicate genotypes unique to males 1 (*bb* homozygote) and 2 (*ab* heterozygote) indicating both must have shared paternity. Mating B: black arrows indicate unique genotypes indicating males 1 and 2 must have a paternity share (cf. Table 3). Paternity for male 3 cannot be excluded (see text).

## RESULTS

## Mating Behaviour

We observed 106 matings at the four sites. In 53 matings, two or more males were amplexed or attempted amplexus with a female at some time during the period of egg deposition (Table 1). The number of males amplexed varied from one to eight. Across all sites, the weighted average frequency of polyandrous matings was 43.5%. Polyandry occurred at all four field sites and in all 3 years indicating that this is not a site- or season-specific phenomenon and is probably, therefore, a general feature of the mating behaviour of this species.

In 22 polyandrous matings where we recorded male position (Kangaroo Gully: 19; Tash's Rock: 3), the focal male was in the conventional, dorsal, inguinal amplexus position and the second male amplexed ventrally. Ventral males held on to the sides of the female between her arms and legs, well in front of the arms of the dorsal male (Fig. 1). However, the cloacae of dorsal and ventral males were approximately apposed. We recorded third males amplexed over the legs of females (Fig. 1), on the sides of females and on the back of, or attempting to slide under, the focal male with other males variously associated with their cloacae at increasingly further distances from the cloaca of the female.

## Paternity Analysis

For both matings, male 1 was focal, male 2 was ventral and male 3 was amplexed over the right leg. The cloacae of males 1 and 2 were in line with the cloaca of the female when eggs were released. The cloaca of male 3 was displaced laterally from the site of egg release.

In Fig. 2 we outline observed genotypes of parents and possible genotypes amongst offspring. In mating A, offspring with the *Gpi*, *Lgsp* genotypes *aa*, *ab* and *ab*, *ab* could be unequivocally assigned to male 2 (Fig. 2, Table 2). Male 3 was unlikely to have fathered any offspring as no offspring with the genotype *bb*, *bb* were detected (expected number of offspring 4.4 if all males have an equal share in paternity). If male 3 is excluded, both males 1 and 2 must have shared paternity as genotypes unique to both were found: for male 1 *aa*, *bb* and *ab*, *bb*; for male 2, as above. However, it is possible that male 3 fathered some offspring with genotypes shared with male 1 (*aa* or *ab*, *bb*). Either way, at least two males must have shared paternity as the genotype *aa*, *bb* cannot come from male 2 but could come from either male 1 or 3. If only males 1 and 2 fertilized eggs, the paternity share does not differ significantly from equality:  $\chi^2_6=1.04$ , NS; for all three males sharing equal paternity:  $\chi^2_6=14.32$ ,  $P<0.05$ ; for only males 2 and 3 sharing paternity:  $\chi^2_6=22.32$ ,  $P<0.005$ .

**Table 2.** Offspring genotypes for frogs from mating A

Genotype	Offspring	Proportion	Possible fathers
<i>aa, ab</i>	6	0.113	2 only
<i>aa, bb</i>	6	0.113	1, 3
<i>aa, bc</i>	11	0.208	1, 2
<i>ab, ab</i>	7	0.132	2 only
<i>ab, bb</i>	8	0.151	1, 3
<i>ab, bc</i>	15	0.283	1, 2
<i>bb, bb</i>	0	0.000	3 only

Two locus (*Gpi, Lgpp-2*) genotypes of parents: female *ab, bb*; male 1 *aa, bc*; male 2 *aa, ac*; male 3 *ab, bb*.

**Table 3.** Offspring genotypes for frogs from mating B

Genotype	Offspring	Proportion	Possible fathers
<i>ab</i>	31	0.492	1, 2, 3
<i>bb</i>	18	0.286	1 only
<i>bc</i>	14	0.222	2 only

Genotypes (*Gpi*) of parents: female *bb*; male 1 *ab*; male 2 *ac*; male 3 *aa*.

In mating B, males 1 and 2 must have had a share in paternity because the *Gpi* genotypes *bb* and *bc* can come only from matings involving these two males (Table 3). The *ab* genotype could come from any of the three males so a share in paternity by male 3 cannot be excluded. If only males 1 and 2 fertilized eggs, the paternity share does not differ significantly from equality:  $\chi^2_2=0.516$ , NS; in contrast, for all three males sharing paternity equally:  $\chi^2_2=9.41$ ,  $P<0.01$ .

## DISCUSSION

Our data show that synchronous polyandry occurred in half of all matings we observed in *C. georgiana* (Table 1). Multiple males remained amplexed throughout egg deposition. There was always one male in inguinal amplexus (focal) with a second male amplexed in a ventral position (Fig. 1). This means the cloacae of focal and ventral males were about equidistant from the site of egg release (Fig. 1). The allozyme data (Tables 2, 3) indicate that at least two males were successful in fertilizing eggs in matings involving three males. However, we cannot exclude the possibility that third males achieved some fertilizations. Assuming that only two males fertilized eggs, the success of focal and ventral males was about equal (Tables 2, 3). D'Orgeix & Turner (1995) also found roughly equal paternity for each of two males in their observations of *A. callidryas*, where both males were in dorsal amplexus.

This is only the second direct demonstration of multiple paternity in anuran amphibians with external fertilization and only the fourth example where it has been shown to be likely (cf. Jennions & Passmore 1993; D'Orgeix & Turner 1995; Laurila & Seppa 1998). However, the mating behaviour of frogs in these four possible

examples is quite different. For *Chiromantis* and *Agalychnis* there are repeated bouts of egg deposition over several hours with multiple opportunities for additional males to attempt amplexus. *Chiromantis* has an arboreal, foam egg mass but eggs of *Agalychnis* are deposited on the surface of leaves (Pyburn 1970; Jennions et al. 1992; D'Orgeix & Turner 1995). For *R. temporaria*, conventional, aquatic, egg masses with multiple paternity apparently arise from free-swimming sperm in the water from males not amplexed with the focal female (Laurila & Seppa 1998). The mating behaviour of *C. georgiana* differs from all previous examples with a short amplexus duration (averaging 23 min, unpublished data), amplexed pairs well separated from each other and eggs deposited in water-filled depressions around ca. 1 cm deep (unpublished data). These differences in mating behaviour suggest very different selective factors may have favoured the evolution of polyandry in different frog species.

How common is multiple paternity in frogs? Anurans do exhibit some features often interpreted as adaptations to the risk of sperm competition. For example, some rhacophorid frogs have exceptionally large testes (Kusano et al. 1991; Jennions & Passmore 1993) which are associated with unusual sperm morphologies (Kuramoto 1996): both standard expectations from sperm competition theory (e.g. Møller 1988; Parker 1993; but see Emerson 1997 for alternative explanations of variation in testis size).

However, the occurrence of wide variance in testis volume, coupled with many observations of mating systems that have the potential to lead to sperm competition and multiple paternity, suggest that these latter two phenomena may be much commoner in anuran amphibians than has been reported. In some groups it may be widespread with several reports of multiple male matings in rhacophorids (e.g. Feng & Narins 1991; Fukuyama 1991; Jennions et al. 1992) and at least two species reported in the phyllomedusine, hylid genus *Agalychnis* (*A. callidryas*: D'Orgeix & Turner 1995; *A. saltator*: Roberts 1994).

Hylids, rhacophorids, ranids and myobatrachids represent phylogenetically distinct lineages in modern frogs (Ford & Cannatella 1993) suggesting convergent evolution of polyandrous mating strategies. The broad phylogenetic occurrence may also mean that polyandry is more common than has been reported in anurans. Few studies of anuran mating systems have specifically sought evidence of this behaviour. For example, it has not previously been reported in *C. georgiana* or *R. temporaria* despite several field studies on both species (Savage 1961; Main 1965; Ayre et al. 1984; Seymour & Roberts 1995; references in Laurila & Seppa 1998).

As Halliday & Verrell (1984) prophetically noted, multiple paternity may be more common in anuran amphibians than reported.

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