

Female polyandry under male harassment: the case of the common toad (*Bufo bufo*)

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Abstract

Several recent studies have demonstrated the occurrence of multiple paternity in anuran amphibians, implying that it is more common than previously thought. However, an adaptive explanation for polyandry in anurans is still lacking. The common toad *Bufo bufo* is an explosively breeding species that releases its eggs in strings. The operational sex ratio (OSR) is male biased, causing strong scramble competition among males; females can even drown through harassment during multiple amplexi. We used microsatellite markers to determine patterns of paternity in natural *B. bufo* populations and experimentally mated individuals (females exposed to either two or six males). Thirty per cent of field-collected and 22% of experimentally produced egg strings were sired by at least two males; all others were sired by a single father. Multiple paternities arose only from multiple amplexi, and we found no indication of fertilization from non-amplexing males, for example through free-swimming sperm. Our results suggest that polyandry in *B. bufo* is likely to occur most often at high population densities, and under the most male-biased OSRs. Moreover, polyandry might be interpreted as being the consequence of females spawning when amplexed by a few males, to avoid the risk of drowning by amplexus with multiple males.

Introduction

In recent decades, evidence has mounted that females reproduce with multiple males in a wide range of animal taxa (Birkhead & Møller, 1998; Kraaijeveld-Smit, 2004). Besides direct female benefits such as nuptial gifts, it has been suggested that females may acquire mates with good genes (Yasui, 1998; Hunt *et al.*, 2004), increase the genetic diversity among their progeny (Jennions & Petrie, 2000) or raise the genetic compatibility between gametes (Garner & Schmidt, 2003; Mays & Hill, 2004). However, higher mating rates increase reproductive success primarily in males, and females may even suffer direct costs from superfluous matings (Johnstone & Keller, 2000; Blanckenhorn *et al.*, 2002). Moreover, an increasing number of recent studies also demonstrates that female benefits seem to be generally lacking (e.g. Byrne & Roberts, 1999, 2000; Lee & Hays, 2004). Therefore, the question as to what benefits females gain from polyandry is often still open.

Among vertebrates, most studies on the evolution of genetic mating systems have so far focused on internally fertilizing birds and mammals. For externally fertilizing groups, parentage studies are available for a range of fish species (for a review see Avise *et al.*, 2002), whereas less is known for the second major group, anuran amphibians (Halliday, 1998). When genetic markers were used, multiple

paternity due to either multiple mating or secondary sperm release was regularly detected (D'Orgeix & Turner, 1995; Laurila & Seppä, 1998; Roberts *et al.*, 1999; Lodé & Lesbarrères, 2004; Vieites *et al.*, 2004). An adaptive explanation for polyandry in anurans, however, is so far lacking. Multiply amplexing males can reduce fertilization success, and multiple paternity does not appear to improve offspring fitness (Byrne & Roberts, 1999, 2000). The only indicator for the frequency in which polyandry occurs in anurans seems to be the operational sex ratio (OSR; Byrne & Roberts, 2004; Lodé, Holveck & Lesbarrères, 2005). It directly influences which sex is competing for access to mating partners (Kvarnemo & Ahnesjö, 1996), and for example at male-biased OSR, male competition as well as probability of female coercion increase (Höglund & Robertson, 1988).

The common toad *Bufo bufo* is an explosively breeding species that is widely distributed across Europe (Gasc *et al.*, 1997). Unlike all other anurans for which polyandry has been confirmed, *B. bufo* eggs are ejected progressively in a pair of long strings containing 1000–8000 eggs (Kuhn, 1994), with male spermatozoa not gaining immediate access to all eggs. The OSR is male-biased (2:1 to 8:1; Davies & Halliday, 1979; Reading, 2001). Females can be subject to coercion and male–male competition, which can lead to several males amplexing one female (Davies & Halliday, 1979; Höglund & Robertson, 1988). Moreover, mating can

be terminally costly for females that drown through the harassment of several competing males (Davies & Halliday, 1979; own observations). The amount of standing genetic diversity as well as the relative effective population size is lower than in other amphibians (Scribner, Arntzen & Burke, 1997; Brede & Beebe, 2004), suggesting a high variance in reproductive success. From this knowledge, we expected polyandry in *B. bufo* to be infrequent and, if present, to depend on the OSR. In this study, we document the occurrence of multiple paternity in egg strings from two natural populations and investigate the effects of OSR in breeding experiments. Also, on the basis of previous knowledge about the mating behaviour of *B. bufo*, we argue that the genetic mating system in *B. bufo* is governed by 'convenience polyandry' (*sensu* Thornhill & Alcock, 1983), from which females gain little benefits from multiple paternity, but which may reduce the probability of drowning from multiple male amplexi.

Materials and methods

Mating behaviour of *B. bufo*

Spawning events in *B. bufo* are highly synchronized, and last a few days to weeks in spring. Most males stay at the pond over the entire reproductive period, whereas females leave the breeding site immediately after spawning. Females are bigger and heavier than males, which develop nuptial pads on their forearms to ensure a tight grip on the female. Males try to seize females as early as possible and often females arrive at the breeding pond already amplexed by a male (Davies & Halliday, 1979; a behaviour that is considered as mate guarding in Wells, 1977). The remaining unmated males search the breeding pond and try to access females by dislodging amplexant males. Attempts of multiple males to mate with a single female occur frequently, and according to Davies & Halliday (1979) only about 20% of males seem to breed successfully.

Field data

Data collections took place at a lake in the northern calcareous Alps of Austria (Schlumsee; for a detailed description see Sztatecsny & Schabetsberger, 2005) in 2002, and at a small artificial lake [Wire Mill Dam (WMD) in Sheffield, UK] in 2004. At Schlumsee, we marked four female *B. bufo* with plastic knee tags and recorded the number of amplexed males during spawning. After spawning, we marked egg strings with adhesive tape for later identification and caught all males that were observed in amplexus with the females. Single toe tips were removed from all females and amplexant males and stored in 96% ethanol. From each female's egg string, we collected the longest possible fragment (50–70 cm) starting from the adhesive tape (egg strings are usually wrapped together, making it almost impossible to gain an entire spawn) and raised the eggs in 5 L plastic tubs (one tub per female) in the field. After hatching, 20 randomly taken tadpoles from each

female were sacrificed in 96% ethanol. At WMD, we sampled six egg strings from unknown parents out of a dense spawning aggregation. We carefully followed each egg string by hand and used scissors to cut out a fragment containing approximately five eggs every 30 cm. Thirty to 50 eggs were raised until hatching in small plastic containers, and hatchlings were preserved as above.

Mating experiments

We collected adults (nine females and 38 males) from a roadside drift fence at Mauerbach, 10 km west of Vienna (Austria), during their spawning immigration on 11 April 2004. We measured snout–urostyle length (SUL) to the nearest millimetre, and body mass (BM) to the nearest 0.1 g. Males were individually marked with numbered knee tags made of adhesive plastic foil. After processing, each female was placed in a tank (1.5 × 1.5 m, 0.6 m deep) filled with aged tap water and furnished with a wooden branch as egg-laying substrate. Four females were each housed with two males, and five females each with six males, respectively. All females spawned within 20 h and the males in amplexus during egg release were recorded. After spawning, we sampled toe tips as described above and released all adults at the breeding site of capture. Again, a sub-sample of eggs was collected along the egg string, raised in plastic tubs and preserved after hatching. To test for the effects of SUL and BM in low OSR tanks, we performed paired Wilcoxon tests between males that were successful and unsuccessful in fathering offspring in SPSS 9.0 (SPSS, 1998). In high OSR tanks, we tested for differences in SUL and BM between tanks performing a Kruskal–Wallis test. As these differences turned out to be non-significant, we compared successful and unsuccessful males using a Mann–Whitney test.

Genotyping and paternity analysis

DNA was extracted from adult and larval tissue samples using an ammonium acetate-based procedure described by Nichols *et al.* (2000). For paternity analysis, we used microsatellite loci *Bbufu11*, *Bbufu13*, *Bbufu15*, *Bbufu49*, *Bbufu62* and *Bbufu65* (Brede *et al.*, 2001). Each 10 µL PCR contained 10–50 ng DNA, 5 pmol (5 µM) of each primer, 0.15 mM of each dNTP, 1.5 mM MgCl₂ and 0.5–1.0 U *Taq* polymerase (Advanced Biotechnologies, Columbia, MD, USA) in the manufacturer's buffer [final concentrations 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween]. PCR amplification was performed using a Tetrad thermocycler (Bio-Rad, Hercules, CA, USA). The PCR profiles were 94 °C for 2 min, followed by 39 cycles of 94 °C for 30 s, the primer-specific annealing temperatures as in Brede *et al.* (2001) for 30 s, and 72 °C for 30 s. Primers were labelled with fluorochromes, and alleles were visualized using an ABI 3730 capillary sequencer and scored with the software ABI Genemapper.

In the experiments where one female encountered six males, we used Cervus 2.0 (Marshall *et al.*, 1998) to probabilistically assign paternity to larvae, based on an

assignment threshold of 80% that a male sired a specific offspring. At WMD, where no parental genotypes were known, we used Gerud 2.0 (Jones, 2005) to reconstruct the maternal genotype and to determine the minimum number of males contributing to the progeny. We then compared offspring with reconstructed parental genotypes. This was necessary as the software offers more than one alternative for the parental genotypes. If an offspring was not compatible with exactly one father, we assigned it to the more successful male. In all other cases where females were known and the number of fathers was small, paternity could be determined through manual, one-by-one assignment of offspring alleles to parental alleles.

Results

We genotyped a total of 57 adult *B. bufo* and 440 of their offspring. The number of alleles ranged from five to 23 (Mauerbach), four to nine (Schlumsee) and five to 10 (WMD) for each locus, respectively (Table 1). In Mauerbach, all loci were in Hardy–Weinberg equilibrium at $P > 0.05$ (as required for paternity analysis in Cervus 2.0) and the exclusion power for all six loci combined was 0.989. WMD and Schlumsee were not tested for Hardy–Weinberg equilibrium because we had insufficient sample sizes of adults, and it was also not required for the paternity assignment.

Field data

At Schlumsee, two females were amplexed by one dorsal male, and two females were sandwiched between two males, one in dorsal and one in ventral position. In three cases, the male in dorsal amplexus fathered all the analysed offspring, whereas in one case 25% of the tadpoles could be assigned to the ventral male (Table 2). The offspring could always unambiguously be assigned to amplexant fathers. Two out of the six WMD samples had a minimum number of two fathers, with the more successful male fathering 59 and 91% of the offspring, respectively. In all other cases, one male was sufficient to explain the offspring genotypes (Table 3).

Mating experiments

Mean male BM was 43.7 g (range: 27.6–65.1 g) and mean SUL was 73.8 mm (range: 66.8–79.5 mm). Females weighed on average 99.7 g (range: 95.5–103.2 g) and mean SUL was 132.2 mm (range: 113.2–169.1 g). No double amplexi occurred in low OSR tanks, and one male fathered all analysed offspring (Table 4). The successful males did not differ from their contestants in mass or size (SUL $n = 4$, $Z = -0.730$, $P = 0.465$; BM $n = 4$, $Z = -1.095$, $P = 0.273$). High OSR tanks did not differ significantly in overall male SUL or BM (SUL $\chi^2 = 2.0$, d.f. = 4, $P = 0.735$; BM $\chi^2 = 8.151$, d.f. = 4, $P = 0.086$). Multiple amplexi with one male in the dorsal and a second male in the ventral amplexus position occurred in three tanks (E5, E7, E9). However, only in E7 and E9 the ventral males were successful in fathering 10 and 33% of the analysed offspring, respectively. The remaining offspring

Table 1 Number of genotyped individuals (n) and number of alleles (No.) for three to six microsatellite loci from three populations of *Bufo bufo* in Austria (Mauerbach, Schlumsee) and the UK (Wire Mill Dam)

Locus	n	No. alleles (size range)	H_O	H_E	Excl
Mauerbach					
<i>Bbufu</i> 11	42	8 (92–130)	0.786	0.779	0.383
<i>Bbufu</i> 13	42	13 (154–200)	0.714	0.872	0.564
<i>Bbufu</i> 15	8	5 (156–188)	0.625	0.667	0.224
<i>Bbufu</i> 49	35	19 (162–208)	0.829	0.912	0.664
<i>Bbufu</i> 62	40	8 (181–201)	0.550	0.818	0.456
<i>Bbufu</i> 65	43	18 (153–250)	0.907	0.929	0.722
Schlumsee					
<i>Bbufu</i> 15	10	4 (162–188)	–	–	–
<i>Bbufu</i> 49	10	8 (162–244)	–	–	–
<i>Bbufu</i> 65	10	7 (168–210)	–	–	–
Wire Mill Dam					
<i>Bbufu</i> 11	155	9 (101–131)	–	–	–
<i>Bbufu</i> 15	145	4 (158–164)	–	–	–
<i>Bbufu</i> 49	88	10 (174–198)	–	–	–

Observed and expected (H_O , H_E) heterozygosities, and exclusion probability (Excl) when one parent is known as calculated from Cervus 2.0 for paternal genotypes at each locus for the Mauerbach population. The greater value of H_E compared with H_O in *Bbufu* 62 was likely the result of a high null allele frequency (0.19) as estimated in Cervus 2.0.

Table 2 Relative paternity for 20 *Bufo bufo* offspring sampled from egg strings from four females at Schlumsee, Austria

Schlumsee	n	M1	M2
Female 1	20	1 (d)	–
Female 2	20	1 (d)	–
Female 3	20	1 (d)	0 (v)
Female 4	20	0.75 (d)	0.25 (v)

Females 1 and 2 were amplexed by one male (M1) in dorsal position (d); females 3 and 4 were amplexed by two males (M1 and M2), one in dorsal and one in ventral position (v).

Table 3 Number of fathers for offspring of six *Bufo bufo* egg strings from Wire Mill Dam (WMD), UK as calculated from Gerud 2.0

	n analysed offspring	n minimum estimated fathers	Proportion of offspring father 1	Proportion of offspring father 2
WMD 1	17	1	1	0
WMD 2	27	2	0.59	0.41
WMD 3	27	1	1	0
WMD 4	15	1	1	0
WMD 5	28	1	1	0
WMD 6	32	2	0.91	0.09

could be assigned to the dorsal males (Table 4). In all other cases, we observed only one amplexant male in dorsal position which sired all analysed embryos (Table 4). The

Table 4 Paternity for offspring of nine *Bufo bufo* females exposed to either two (E1–E4) or six males (E5–E9)

Pair	<i>n</i>	M1	M2	M3	M4	M5	M6	<i>n</i> confidence > 80%	<i>n</i> confidence < 80%
E1	32	0	1 (d)	–	–	–	–	32	0
E2	32	1 (d)	0	–	–	–	–	31	1
E3	21	0 (v)	1 (d)	–	–	–	–	15	6
E4	26	0	1 (d)	–	–	–	–	23	3
E5	17	0	0	0	0	0 (v)	1 (d)	17	0
E6	7	0	0	0	1 (d)	0	0	6	1
E7	20	0	0	0	0.90 (d)	0	0.10 (v)	10	10
E8	32	0	0	0	0	1 (d)	0	11	21
E9	27	0	0	0.66 (d)	0	0	0.33 (v)	0	27

n, number of genotyped offspring; d, male in dorsal amplexus position; v, male in ventral amplexus position. Males are ranked in descending order by snout–urostyle length.

seven males successful in fathering all or part of the analysed offspring were significantly smaller than the unsuccessful males ($Z = -2.10$, $n = 30$, $P = 0.033$) and did not differ from their contestants in BM ($Z = -1.005$, $n = 30$, $P = 0.315$).

Discussion

Polyandry in the common toad was about as frequent as in other anurans (Laurila & Seppä, 1998; Roberts *et al.*, 1999; Lodé & Lesbarrères, 2004). We detected multiple paternity both in naturally breeding populations and under experimental conditions (restricted, however, to high OSR tanks). In all cases, multiple paternities occurred through multiple amplexi, and we were unable to detect any indication of fertilization through free-swimming sperm.

Our results suggest that the genetic mating system of the common toad is largely shaped by male density and OSR during explosive breeding events. Both parameters can vary among and within populations (Arak, 1983). At low densities, males attract females through advertisement calls (Höglund & Robertson, 1988); male–male competition is probably low, and so is the proportion of multiple amplexi. At higher densities and during the peak of the breeding season, intrasexual competition increases, and males switch to energetically more costly active searching behaviour (Arak, 1983; Höglund & Robertson, 1988). Given that the chance of success in a second mating is low, selection should therefore favour males that achieve an early mating. This might explain why males do not release any females, even when a large number of additional competitors attempt amplexus. Females eject egg strings in consecutive pulses while moving around slowly, and we found no evidence for fertilization through free-swimming sperm. Therefore, sperm and egg release must be timed concordantly to ensure fertilization, which is probably better achieved by a dorsal than a ventral male, through the detection of female abdominal movements such as oviductal contractions. Moreover, dorsal males seem to increase fertilization efficiency by creating a basket with their hind legs in which they gather the eggs (C. Reading, pers. comm.). However, paternity analysis was restricted to a small proportion of eggs laid by each female (*c.* <2%). Given this, the frequency of multiple

paternity (including fertilization by stray sperm) was potentially biased and the number of fathers could have been underestimated.

Female common toads are larger than males, despite a mating system in which being a large male might arguably be an advantage. However, there is only weak evidence that mating in *B. bufo* is size dependent (Davies & Halliday, 1979; Höglund & Robertson, 1987; Höglund, 1989), and male size also seems unrelated to offspring fitness (Semlitsch, 1994). Indeed in our experiments, successful males were smaller than unsuccessful ones. We have no clear explanation for this finding; however, effects of male size may be depending on the time available for male–male competition (Höglund, 1989), which was comparably short in our experiments. For females, being amplexed by several males may be terminally costly. Besides the production of a large number of eggs, large body size may increase the ability to survive multiple amplexi. As females are unable to dislodge unwanted males, they have little opportunity for mate preference (Davies & Halliday, 1979). One option for female choice would be to resist spawning and to expose themselves to unmated males that may attempt to displace the amplexant male. Under such circumstances, resistance is considered equivalent to preference, and the most persistent male will be the preferred one (Kokko *et al.*, 2003). However, the costs of mating merely depend on the number of males a female mates with. Thus, the probability of multiple amplexi rises with resistance time for spawning, and a female should accept mating when the costs of resistance exceed the costs of mating ('convenience polyandry'; Thornhill & Alcock, 1983; Lee & Hays, 2004). In *B. bufo*, this might be reached when a female is amplexed by a second male, matching with the observed frequency of polyandry as well as mating observations.

In summary, sexual selection in the common toad seems to be driven by direct costs rather than direct or indirect benefits. At present we have no evidence that multiple paternity is advantageous to females; it largely results from male coercion. Wells (1977) has considered the common toad's mating system analogous to that of yellow dung flies, *Scatophaga stercoraria*, which was among the first species for Parker (1979) to demonstrate the importance of antagonistic coevolution under sexual conflict. However, our

findings rather suggest that females behave indifferently under male harassment, and support recent models of sexual conflict that predict arms races as a consequence of sexual antagonistic coevolution to be less common than previously thought (Härdling & Smith, 2005; Rowe, Cameron & Day, 2005).

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