'IN SITU' PREVENTION OF ANURAN FERTILIZATION – A SIMPLE METHOD FOR THE DETECTION OF SPERM COMPETITION WITH POTENTIAL FOR OTHER APPLICATIONS

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Sperm competition is recognized as an integral component of sexual selection, shaping life-history characteristics such as body size, morphology, physiology and behavior (Birkhead and Parker 1997). Sperm competition occurs when there is competition between the ejaculates of different males for the fertilization of a given set of ova (Parker, 1970). Therefore sperm competition can be shown by detecting mixed paternity using molecular methods (Queller et al. 1993; Roberts et al. 1999), by direct observation of multiple male - single female copulations (Jennions and Passmore 1993; Birkhead and Parker 1997) and by comparing relative testis sizes and sperm traits among and within species based on sperm competition theory (Harcourt et al. 1981; Byrne et al. 2002). However, despite the relatively high number of proposed methods it remains difficult to assess sperm competition in external fertilizers, and our knowledge of the occurrence of sperm competition in externally fertilizing anuran taxa has remained scarce (Halliday 1998).

In the externally fertilizing anurans, the production of offspring often numbering in the hundreds or thousands may render molecular paternity analyses uneconomical. Similarly, comparative methods using testis sizes and sperm traits require large sample sizes (sometimes more than a hundred) of sacrificed animals (Kusano et al. 1991; Jennions and Passmore 1993). This method is also problematic because it does not provide direct evidence for sperm competition (Birkhead and Parker 1997). Behavioral observations (e.g., Fukuyama 1991; Kusano et al. 1991; Kaminsky 1997) also share the latter burden.

In order to demonstrate the occurrence of sperm competition in a rhacophorid frog, Jennions and Passmore (1993) proposed a simple method for the documentation of sperm release by peripheral males in multiple male amplexes. Before oviposition, they placed the amplexed male's lower body in a plastic bag (20 x 20 cm) and let the pair spawn in the presence of peripheral males. After embryonic development they counted hatching larvae and unfertilized eggs. In control treatments, where peripheral males were excluded, fertilization success was zero, whereas in the experimental trial, where peripheral males were present, hatching success was nearly 37%. They concluded that plastic bags act as effective condoms, and that peripheral males sired the tadpoles in experimental treatments. Their method, however, has some substantial weaknesses: Jennions and Passmore only performed two controls and one experimental trial, thus they did not test their method thoroughly for repeatability. They also noted that the plastic bag might have affected the rate at which peripheral males came into close contact with the female's cloaca, possibly reducing fertilization success. Furthermore, it is inevitable that the primary male would be hampered with its lower body completely wrapped in a 20 x 20 cm plastic bag, presumably leading to altered behavior and a strongly decreased ability to keep peripheral males away from the female.

We propose here an alternative technique that overcomes these problems by using

condoms instead of plastic bags. Condoms were washed with soap before use to remove the lubricating gel, which could adversely affect study animals. After rinsing soap residue off with water, two holes were cut opposing each other in the condom. The size of the hole depended on the thickness of the proximal end of thighs of males, as it must prevent sperm leakage but not compress the blood vessels of the legs. Handling and fixing of the condom was easier when the condom was not unrolled completely. Once these preparations were completed, one person held the condom and stretched it open so that the holes for the legs became wide and the other person could easily pull the hind legs of the male one by one through the holes and smooth out the condom on the abdomen. The condom was fastened with two 20 cm long pieces of yarn bound as slings around the abdomen covered by the condom, one just under the fore-limbs and another above the hind-limbs and the cloaca (Fig 1). The slings were tightened enough to prevent the condom from slipping down the body.

FIGURE 1

Amplexed pairs of *Bufo bufo* were hand-collected from a breeding-site in the Pilis Mountains (47º42' N, 19º01' E) 25 km to the north of Budapest, Hungary on five occasions in mid-April 2002. Pairs were transported in covered plastic boxes to the Ecology Laboratory at Eötvös Loránd University, Budapest, where trials were conducted within 24 hours of capture. Two inflatable plastic wading pools (120 cm x 120 cm, 60 cm deep) filled to a depth of 25 cm with aged tapwater were used as artificial egg-deposition sites.

We ran one control (no condom) and one experimental trial (with condom) at a time. Treatments were assigned randomly to wading pools. A total of five replicates were performed for each treatment. Pools were washed thoroughly with aged tapwater between successive trials. Treatments were terminated following egg deposition, which occurred within 16 hours in all cases. Animals were then removed and returned to the site of their collection, and egg strings were placed into uncovered boxes (55 cm x 35 cm, 30 cm deep) filled to a depth of 3

cm with aged tapwater. Eggs were stored for approximately three days at room temperature, so that embryonic development advanced to Gosner stage 18 (Gosner 1960). After this time fertilized and unfertilized eggs could be readily distinguished. In clutches taken from experimental trials we counted all eggs, but estimated egg number in the controls only to the nearest 100, as control treatments were only applied to test whether the experimental design grossly modified reproductive behavior and/or fertilization success. Embryos were reared until hatching and were then released at the site of their parents' collection. Experimental trials were video recorded to survey if the condom affected the behavior of the amplexed pair and whether it remained set in its place.

Bufo bufo females ($n = 5$) deposited on average 5305 eggs in experimental trials ($n = 5$; SD = 1121) and 5360 eggs in control treatments ($n = 5$; SD = 1064). We found no difference in number of eggs deposited between experimental trials and control treatments (Student *t*-test, $t_8 = 0.08$; $P = 0.938$). Average fertilization success was 0.303% (SD = 0.293) in experimental trials and 93.2% (SD = 8.55) in control treatments, and this difference was strongly significant (Mann-Whitney $U = 0.00$; $P<0.01$). In two cases the pair in the experimental trial started egg laying first, in three cases it was the pair in the control. There was no observable change in the position of condoms in any of our experimental replicates. Video records confirmed that the males did not try to remove the condoms, that the condoms remained in their position and that the males remained in amplexus until egg deposition was complete. After treatments, condoms contained an opaque fluid that we checked for spermatozoa under a light microscope, at 800x magnification. We found actively swimming spermatozoa in high numbers in the condoms of all experimental replicates.

Birkhead and Parker (1997) suggested two ways to detect sperm competition in internally fertilizing species: 1) by direct observation of multiple male mating assemblages, and 2) by detecting mixed paternity. However, it is important to note that both methods have limitations.

In the first, copulation does not always result in insemination. In the second, it is possible that secondary males do not sire any progeny. Birkhead and Parker (1997) concluded the combined use of both methods is desirable despite the difficulties in carrying out such studies. The same requirements should also apply to externally fertilizing species with the modification that, instead of multiple copulations, sperm release by multiple males targeting the same set of eggs must be detected. With our method it is possible to satisfy both of Birkhead and Parker's (1997) requirements without the use of molecular markers. If the focal male of a pair is equipped with a condom and the resulting clutch contains a high ratio of fertilized eggs, sperm competition can be assumed to occur under natural conditions as sperm from males other than the focal male must have achieved those fertilizations.

The testing of our method showed the following: (1) Fertilization success was negligible in all experimental trials, indicating that our method is effective in the retention of sperm of the amplexed male; (2) Among experimental trials we found low variance in fertilization success, showing our method to be highly repeatable; (3) Low fertilization success was due solely to the inhibition of fertilization by condoms, as average fertilization success in control treatments was comparable to that found in natural populations (A. Hettyey, personal observation) (4) Video records showed that the condoms applied as described stayed in position throughout the trials, allowing males to use their hind-legs without any difficulty. The condoms fitted *Bufo bufo* males tightly, clinging onto the males' abdomen and clearly not hanging over the females' cloaca. Thus they would not have prevented the sperm of other males from fertilizing emerging eggs; (5) High numbers of live spermatozoa were found in the condoms after termination of experimental trials, again documenting the efficacy of condoms in retaining the ejaculate. We note, however, that condoms appropriate for use in *Bufo bufo* and other large and medium-sized species may not be applicable for small frogs and toads. In this case pharmaceutical finger cots can be used instead of condoms (A. Hettyey, unpublished data).

The fact that our method enabled the collection of ejaculates suggests other applications of this technique. For instance, artificial fertilization is a widely used method in a variety of studies (e.g., Berger & Rybacki 1992; Semlitsch 1994; Rakitin et al. 1999). In anurans the traditional method of obtaining sperm for artificial fertilization is to sacrifice and dissect the males, squash their testes and then prepare sperm-suspensions (Berger et al. 1994). Such sacrifice may bring up ethical and conservation problems, possibly constraining the researcher's ability to obtain sperm from some populations or species. Gutleb et al. (2001) have shown that water that came into intensive contact with latex gloves can damage amphibian larvae. However, in our experiments, animals did not seem to be harmed by the condoms and the condoms contained live, actively swimming spermatozoa after trials. Consequently, we conclude that our technique allows the collection of sperm without killing or damaging males and allows the use of ejaculated sperm (which is superior in many aspects to sperm from dissected testes (Wilson et al. 1998). Another possible application is the testing of sperm competition theory suggesting differences among and within males in quality and quantity of sperm spent on distinct ejaculates (for a review, see Parker 1998). Predictions offered by theory have not been tested in anurans so far, probably due to the lack of an appropriate method of ejaculate sampling. As our technique retains almost the entire ejaculate, it offers the possibility to examine actual ejaculates and to compare them among or within individuals. Potential applications of our method are the detection of mating preferences at the level of ejaculate expenditure and toward male quality estimation based on sperm characteristics. Although this might be accomplished using the traditional dissection approach, our method collects only ejaculated sperm and avoids collecting samples contaminated with cells not directly involved with fertilization.

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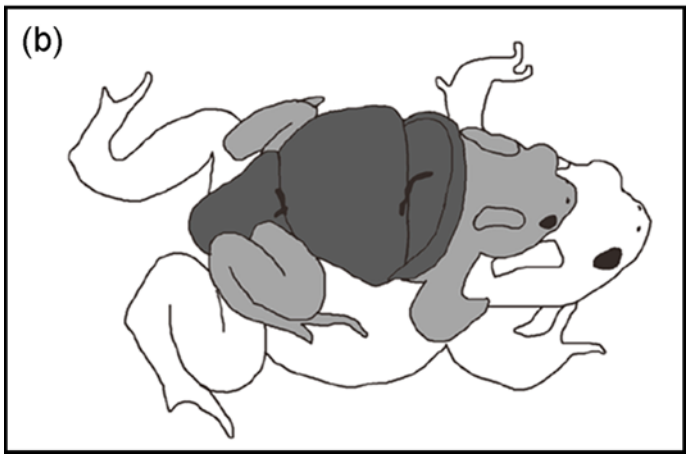
Legend to figure

Figure 1: (a) Photograph on an amplexed *Bufo bufo* male equipped with condom. (b) Diagrammatic interpretation of photograph in (a): Female: white, male: light grey, condom: dark grey.


(a)


(b)