



## Simultaneous polyandry increases fertilization success in an African foam-nesting treefrog

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In many animals females mate with multiple males during a single breeding season (polyandry), but the benefits of this mating system remain poorly understood. One hypothesis is that polyandry ensures the fertilization of a female's ova (fertilization insurance hypothesis). We tested the fertilization insurance hypothesis in a natural population of African foam-nesting treefrogs, *Chiromantis xerampelina*, a species lacking male contest competition and in which females routinely mate with multiple males. We observed matings involving from one to 12 males and found that fertilization success was positively correlated with the number of mating males, but was unaffected by variance in clutch size or ambient temperature. Variance in fertilization success was also unrelated to the body size ratio of mating pairs. Critically, females that mated with more males also produced more tadpoles. These findings provide the first evidence that polyandry can benefit female frogs by increasing fertilization success and offspring production. We propose that fertilization insurance may account for the high incidence of simultaneous polyandry in frog species that use foam nests during breeding.

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In many animals, females mate with multiple males during a single reproductive period (polyandry; Birkhead 2000). Why this mating strategy is so taxonomically widespread is currently one of the most compelling, but least understood, questions in evolutionary biology (Simmons 2005). In some cases, males may force females to copulate against their best interests (Thornhill 1980; Rice et al. 2006). However, in cases where females actively solicit matings the implication is that polyandry benefits females (Zeh 1997). Theoretically, polyandry can benefit females indirectly, by providing genetic benefits for offspring (Zeh & Zeh 1996; Jennions & Petrie 2000; Yasui 2001; Fisher et al. 2006), or directly, by providing material benefits (Zeh & Smith 1985; Birkhead 1995). One potential direct benefit of polyandry is that mating with multiple

males ensures the fertilization of a female's ova (fertilization insurance hypothesis; Parker 1970; Walker 1980; Birkhead & Parker 1997).

Evidence to suggest that polyandry acts to ensure fertilization success has been obtained for several taxa with internal fertilization, including insects, salamanders, birds and mammals (Gibson & Jewell 1982; Ridley 1988; Wetton & Parkin 1991; Krokene et al. 1998; Osikowski & Rafinski 2001). What remains to be determined is whether fertilization insurance is also a common benefit of polyandry in groups with external fertilization (Petersen 1991; Levitan 2005; Smith & Reichard 2005). In general, external fertilization is a risky reproductive mode because high fertilization success often requires that the sexes are closely aligned during gamete release, gametes are released synchronously and gamete union is unhindered by physical barriers (Bourne 1993; Shapiro & Giraldeau 1996; Levitan 2005). In addition to these potential problems, external fertilization exposes gametes to environmental variables that can reduce their viability. For example, at low temperatures sperm motility can be substantially slowed resulting in reduced fertilization success (Vladic & Jatrvi 1997). External

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fertilizers typically also produce large clutches, thereby reducing fertilization success by males with finite sperm supplies during successive matings (Shapiro & Giraldeau 1996). Given the high risk of suboptimal fertilization success inherent in the external mode of reproduction, it seems probable that polyandry will have evolved as a fertilization insurance mechanism in many taxa.

Among anuran amphibians (frogs) simultaneous polyandry has been reported in at least 13 species from five families (Coe 1967, 1974; Pyburn 1970; Feng & Narins 1991; Fukuyama 1991; Roberts 1994; Kasuya et al. 1996; Kaminsky 1997; Byrne & Roberts 1999). However, the fertilization insurance hypothesis has been tested in only one species, the West Australian myobatrachid frog *Crinia georgiana* (Byrne & Roberts 1999). Contrary to prediction, female *C. georgiana* incur significant reductions in fertilization success when they mate with multiple males. The most likely explanation for this relationship is that competing males interfere with egg laying or the ability of rival males to assume optimal mating positions or sperm release cycles (Byrne & Roberts 1999). This cost has also been found in some birds where conspecifics interact intensely (Koenig 1982; Davies 1992). In many frogs, breeding occurs at high density and males fight violently to grasp females during spawning (Pyburn 1970; D'Orgeix & Turner 1995; Kaminsky 1997; Byrne et al. 2002). Therefore, reduced fertilization success may be a common cost of simultaneous polyandry in anurans. However, not all frogs with simultaneous polyandry experience intense male–male competition during spawning (Halliday 1998). For instance, in several species of frogs that build foam nests from the families Rhacophoridae and Leptodactylidae, males involved in multiple-male spawning do not physically compete to grasp females. Instead, males systematically position themselves around foam nests and show behaviours (e.g. muscle convolutions) that are associated with sperm release. In these frogs, it seems highly probable that simultaneous polyandry might increase fertilization efficiency (Kusano et al. 1991).

Of the foam-nesting species where simultaneous polyandry has been reported, the African foam-nesting treefrog *Chiromantis xerampelina*, has the highest frequency of multiple-male spawning (93%), as well as the highest mean number of males ( $N = 5$ ) involved in a spawning (Jennions & Passmore 1993). Unpaired males also contribute sperm during spawning events. Using a sterile male experiment, Jennions & Passmore (1993) excluded the sperm of paired males in multimale spawnings and showed that unpaired males can successfully gain fertilizations. The aim of our study was to determine whether simultaneous polyandry in *C. xerampelina* leads to a significant increase in fertilization success, and a corresponding increase in the number of offspring (tadpoles) produced.

## METHODS

### Study Species

*Chiromantis xerampelina* is a large (24–36 mm snout–vent length), rhacophorid frog, endemic to regions of

mesic savannah in southern Africa. The species is a nocturnal breeder, with mating activity occurring during the wet summer months (October–February). Males gather in trees surrounding temporary water bodies and produce irregular advertisement calls to attract females (P. G. Byrne, personal observation). The males aggregate in groups along tree branches but they do not compete aggressively for call sites (P. G. Byrne, personal observation). When females enter breeding trees they are quickly grasped by a male, who is subsequently carried onto a branch where other males are usually gathered (P. G. Byrne, personal observation).

Mating is arboreal with females laying eggs in foam nests that are constructed on branches overhanging water. With the assistance of males, a female will build her nest by releasing a cloacal mucus, along with water expelled from her bladder, and whipping the blend into a lather by churning her back legs (Seymour & Loveridge 1994). Females release eggs into the nest during discrete bouts of foaming, and also take extended breaks (approximately 20–40 min) to return to water bodies and refill their bladders (unpublished data). In total, nest construction takes approximately 5–6 h (P. G. Byrne, personal observation.). Once nests have been constructed, embryos develop rapidly, and after approximately 5 days tadpoles wriggle free from the foam and drop into the water below. Although matings can involve a female and a single male (monogamous matings), they usually involve females and multiple males (polyandrous matings; Jennions et al. 1992). These polyandrous matings are the outcome of unpaired calling males joining a pair during nest construction. It is possible that unpaired males participate in nest construction because they churn their back legs in synchrony with the amplexant pair (unpublished data).

### Study Site

Fieldwork was conducted at Hans Merensky Nature Reserve (24–25°38'S, 31–22°40'E, 462 m above sea level), 70 km northeast of Tzaneen, Limpopo Province, South Africa. The breeding site was a temporary pond (Tsonga Kraal Dam) surrounded by native bushland dominated by mopane trees, *Colophospermum mopane*. The dam filled after torrential rainfall on 18 November 2006, and on 19 November 2006 male *C. xerampelina* entered the site and began calling. Breeding commenced the following evening when females entered the site. Most breeding took place on tree branches overhanging the pond, but some matings took place on emergent vegetation at the water's edge. Data were collected between 1800 and 0700 hours from 19 November 2006 to 14 February 2007, during which time ambient temperature ranged between 19 and 28.5 °C. (mean 24 °C). The study was conducted with permission of the University of Witwatersrand Ethics Committee.

### Behavioural Observation of Matings

We located gravid females by conducting nightly searches of the trees surrounding the pond. We followed

females until they were grasped by a male and then followed the pair until they commenced nest construction. The mating event was then continuously observed so that we could record the number of males that came into bodily contact with the foam nest. Males in contact with the nest were closely aligned with the amplexant pair and also displayed behaviours (e.g. muscle convulsions) typically associated with sperm release. Therefore, we could confidently record every male that participated in a group spawning. After females finished nest construction, and mating assemblages broke up, all the frogs involved were collected by hand and stored in plastic zip-lock bags. We then measured their snout–vent length ( $\pm 1$  mm) using a plastic ruler. Body size was measured as length rather than mass because the body mass of frogs can vary substantially depending on hydration state. No frog was kept in a plastic bag for longer than 15 min and every frog was returned to the nest site where it was captured. Females, which were spent, rapidly left the breeding site but males either remained in close proximity to the nest site or moved into adjacent trees.

### Collection of Nests and Determination of Clutch Fertilization

Approximately 24 h after nests were constructed, we collected them and placed them in wire-mesh frames suspended within 10-litre plastic buckets containing 5 litres of fresh water. Each bucket was covered with a plastic bag to maintain a humid environment and prevent eggs from desiccating. The buckets containing nests were then stored in a room approximately 20 m from the study site. The room was air conditioned with an ambient temperature of between 26 and 31 °C. Approximately 72 h after nests were constructed, they were moved to a different 10-litre bucket and gently agitated with tap water to separate developing embryos and unfertilized eggs from congealed foam. For each nest we counted total egg number and scored the percentage of eggs fertilized.

### Determination of Offspring Survival

In many animals, embryos can fail during early stages of development, so fertilization success will not always equate with the final number of offspring produced, which is the ultimate measure of a female's reproductive success (García-González 2008). Therefore, to determine the rate of embryo survivorship in *C. xerampelina*, embryos were kept in large plastic tubs filled with tap water until they matured into tadpoles. After 5 days, which is the approximate time taken for tadpoles to hatch from a nest in nature, we counted the tadpoles in each clutch. Tadpoles were then released into the dam directly underneath where the original nest sites were constructed. If water was no longer present underneath the nest site, tadpoles were released at the dam's edge. After tadpoles were released, their survivorship would have been determined by the intensity of natural selection operating at the study site. To determine whether fertilization success

reliably predicts tadpole survival in *C. xerampelina*, we used a linear regression model.

### Effect of Number of Males, Temperature and Clutch Size

In addition to counting males involved in a spawning, we measured temperature at the nest site and the total number of eggs released (clutch size). Temperature was recorded because sperm motility and/or viability might be temperature dependent, and this could influence fertilization success (Vladic & Jatrvi 1997). Clutch size was recorded because larger clutches may have reduced fertilization success if male *C. xerampelina* limit the quantity of sperm they allocate to each mating (Shapiro & Giraldeau 1996). To test the relative importance of number of spawning males, temperature and clutch size on the proportion of a clutch fertilized, we used a multiple regression model. For analysis, percentage data were arcsine transformed. A multiple regression model was also used to test the relative importance of number of spawning males and temperature on the total number of eggs fertilized. Clutch size was not included in this model because it would have been autocorrelated.

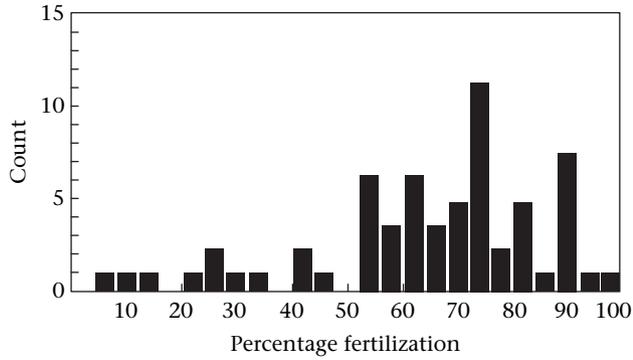
### Effect of Body Size Ratio

For external fertilizers, the proximity of the sexes to each other during gamete release can significantly influence fertilization success. In several frog species, specific body size ratios are required to optimize fertilization success (Robertson 1990; Bourne 1993). If this is the case in *C. xerampelina*, the relationship between body size ratio and fertilization success should be curvilinear. Therefore, for matings that involved a single female and a single male, we calculated body size ratios for each pair (male snout–vent length/female snout–vent length) and used a polynomial (quadratic) regression to test for a curvilinear relationship between body size ratio and fertilization success. We also used linear regression to test whether fertilization success was independently influenced by the body size of either sex.

## RESULTS

### Fertilization Success and Offspring Survival

On average, the fertilization success of clutches  $\pm$  SE was  $63.51 \pm 2.97\%$ , but there was considerable variance between clutches (range 7.40–97.25%,  $N = 55$ ; Fig. 1). For eggs that were fertilized, tadpole survivorship after 5 days of development was very high (mean survival  $\pm$  SE =  $92.62 \pm 0.93\%$ , range 72.27–100%,  $N = 55$ ). This resulted in a very strong positive correlation between the number of fertilized eggs and the number of offspring produced (linear regression:  $R^2 = 0.98$ ,  $F_{1,54} = 2637.1$ ,  $P < 0.0001$ ; Fig. 2).



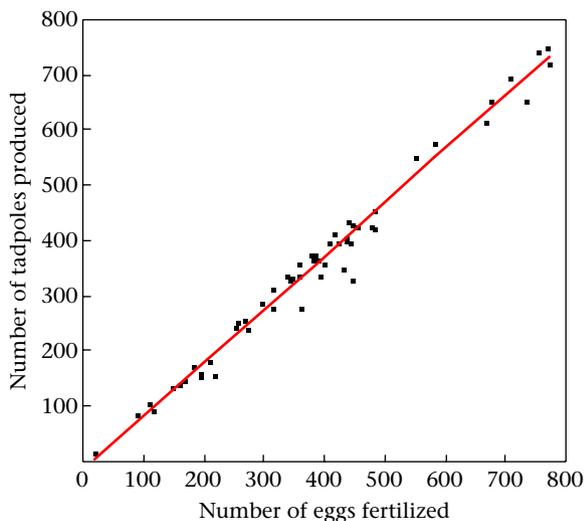
**Figure 1.** Histogram showing the distribution of percentage clutch fertilization in the foam-nesting treefrog *Chiromantis xerampelina*.

### Effect of Number of Males, Temperature and Clutch Size

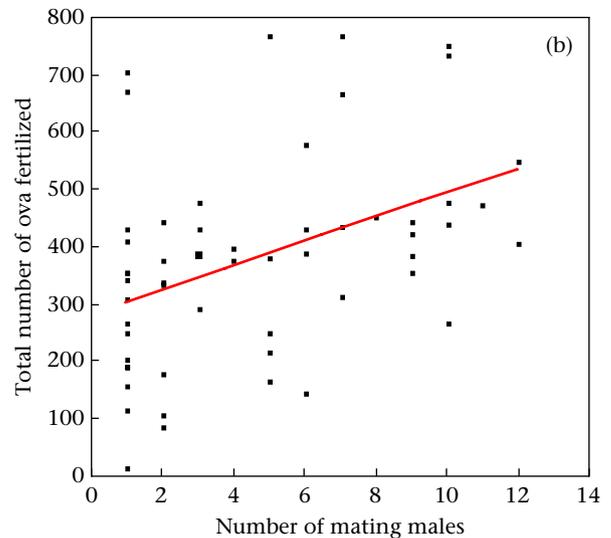
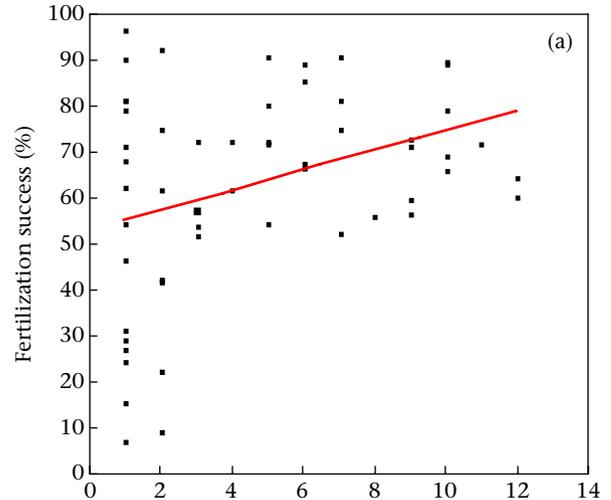
Variation in the percentage of eggs fertilized within a clutch was positively and significantly associated with the number of males involved in a mating (Fig. 3a), but was not significantly influenced by either variation in clutch size (range 270–930 eggs) or ambient temperature (range 19–28.5 °C; multiple regression: whole model: % clutch fertilized:  $R^2 = 0.111$ ,  $F_{3,54} = 2.13$ ,  $P = 0.10$ ; number of males:  $F_{2,54} = 5.69$ ,  $P = 0.02$ ; clutch size:  $F_{2,54} = 0.06$ , NS; temperature:  $F_{2,54} = 0.29$ , NS). Similarly, variation in the total number of eggs fertilized was positively and significantly associated with the number of mating males (Fig. 3b), but was unrelated to variation in ambient temperature (multiple regression: whole model:  $R^2 = 0.2$ ,  $F_{3,54} = 6.60$ ,  $P = 0.002$ ; number of males:  $F_{2,54} = 11.22$ ,  $P = 0.001$ ; temperature:  $F_{2,54} = 1.84$ , NS).

### Effect of Body Size Ratio

For matings that involved one male and one female ( $N = 16$ ), variance in fertilization success (mean  $\pm$



**Figure 2.** Relationship between percentage clutch fertilization and number of offspring (tadpoles) produced in the foam-nesting treefrog *Chiromantis xerampelina*.

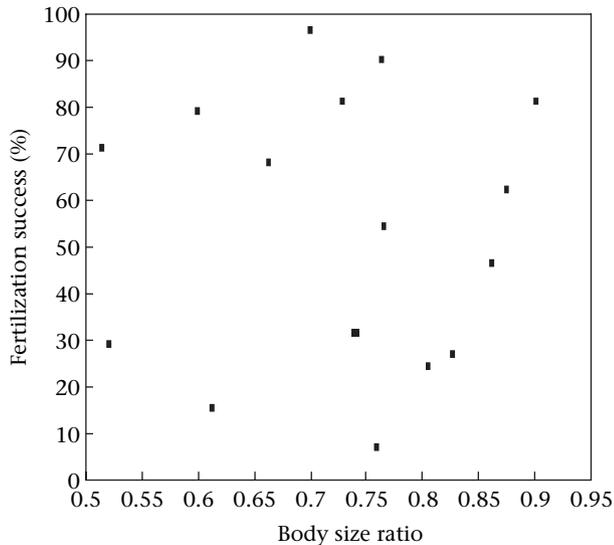


**Figure 3.** Number of males involved in group spawnings in the foam-nesting treefrog *Chiromantis xerampelina* plotted against (a) percentage clutch fertilization and (b) the total number of eggs fertilized.

SE =  $53.74 \pm 7.22$ , range 7.40–97.25%) was unrelated to variance in the body size ratio of mating pairs (quadratic regression:  $R^2 = 0.0004$ ,  $F_{1,15} = 0.0031$ ,  $P = 0.99$ ; Fig. 4). Variance in fertilization success was also unrelated to variation in the body size of males (linear regression:  $R^2 = 0.035$ ,  $F_{1,15} = 0.51$ ,  $P = 0.48$ ) or females (linear regression:  $R^2 = 0.032$ ,  $F_{1,15} = 0.46$ ,  $P = 0.50$ ).

## DISCUSSION

Our study tested the hypothesis that female foam-nesting treefrogs increase fertilization success by mating with multiple males. As predicted, females that mated with more males experienced higher fertilization success, in terms of both the proportion of a clutch fertilized and the total number of eggs fertilized. Polyandrous females also had higher reproductive success because there was a strong positive correlation between fertilization success and



**Figure 4.** Relationship between body size ratio (male snout–vent length/female snout–vent length) and fertilization success in matings involving one male and one female in the foam-nesting treefrog *Chiromantis xerampelina*.

offspring survival. These results provide evidence that polyandry benefits female *C. xerampelina* by ensuring fertilization success and increasing offspring production.

For females that mated with single males, fertilization success was generally less than 60%, which is substantially lower than the level of fertilization (90–95%) normally found in monogamous frog species (e.g. Davies & Halliday 1977; Howard 1978; Kruse 1981; Ryan 1985). Previous studies reporting low fertilization success in frogs have linked this problem to mismatches in body size between mating pairs (Licht 1976; Davies & Halliday 1977; Krupa 1988), but we found no evidence for such a relationship in *C. xerampelina*. We also found no evidence that variance in fertilization success was linked to fluctuations in ambient temperature. We presumed this might occur if temperature affected sperm motility and/or viability, as is the case in other external fertilizers (e.g. fish, Vladic & Jatrvi 1997).

One explanation for variable fertilization success in *C. xerampelina* is that males vary in the intrinsic quality of their sperm, and this affects their fertilization efficiency (intrinsic male quality hypothesis; Yasui 1997). It is now well established across a variety of animals that sperm traits, such as flagella length, can significantly influence a male's fertilization success (García-González 2008). Moreover, in some insect species heritable sperm traits known to influence fertilization efficiency vary between males (García-González 2008). Fertilization success in *C. xerampelina* could also vary because of high levels of genetic incompatibility (genetic incompatibility hypothesis; Zeh 1997). For example, in the Australian urchin *Helicidaris erythrogramma* interactions between male and female genotypes significantly influence fertilization success (Evans & Marshall 2005). To test critically whether variance in fertilization success in *C. xerampelina* is linked to 'intrinsic sire effects' or 'genetic incompatibility' further experiments will be required. One way to distinguish

between these effects would be to use in vitro fertilization procedures to conduct a North Carolina II maternal half-sib breeding experiment whereby gametes from equal numbers of males and females are crossed in every conceivable pairwise combination (Lynch & Walsh 1998). If a subset of males gained high fertilization success across all females, this would provide support for the intrinsic male quality hypothesis. Conversely, if there was a significant interaction between male and female genotypes (sire  $\times$  dam interactions) this would implicate genetic incompatibility as an important factor underlying variance in fertilization success.

Another possible explanation for low fertilization success in *C. xerampelina* is that encounter rates between sperm and eggs are limited. There could be several reasons for low gamete encounter rates in this frog. One possibility is that the viscous foam used during nest construction inhibits gamete fusion. However, this seems unlikely because Australian myobatrachid frogs that use foam nests have smaller testes relative to body mass than closely related 'nonfoaming' species (Byrne et al. 2002). The implication of this relationship is that foam nesting actually increases fertilization efficiency. A more plausible explanation for variance in fertilization success is that individual males frequently ejaculate insufficient sperm. This could occur if males practise sperm economy and distribute limited sperm over successive matings (Shapiro & Giraldeau 1996). The pressure for sperm economy in *C. xerampelina* is likely to be high because individual males frequently take part in multiple spawning events, within and between nights (P. G. Byrne, personal observation). If females vary in their quality, sperm-limited males might also actively refrain from fully investing in matings that are unlikely to yield large returns (Marconato et al. 1995). Such variable sperm allocation strategies have been found to underlie suboptimal fertilization rates in certain species of fish (Shapiro et al. 1994).

Sperm limitation could also occur in *C. xerampelina* if males tailor their ejaculate investment according to the risk and intensity of sperm competition (Ball & Parker 1996). Theoretically, males should reduce sperm investment in matings involving two or more competitors (Parker et al. 1996). If paired males perceive sperm competition to be intense (more than two males), but competitors do not release sperm, paired males may release too few spermatozoa. Reduced ejaculate size in response to intense sperm competition remains to be shown in frogs (Byrne 2004); however, this response has been found in a diversity of other animals (e.g. insects: Schaus & Sakaluk 2001; mammals: delBarco-Trillo & Ferkin 2006). To test for strategic male ejaculation in *C. xerampelina*, it would be necessary to expose males to various conditions of sperm competition risk and intensity and collect their ejaculates, which can be achieved by covering a male's cloacal region with a plastic or latex receptacle (Jennions & Passmore 1993; Hettyey & Török 2005).

Variable fertilization success in *C. xerampelina* might also be linked to sperm depletion (Warner et al. 1995). This is a limitation that appears to explain suboptimal fertilization efficiencies in many animals (Wedell et al. 2002), including frogs (e.g. Robertson 1990; Bourne 1993). Sperm

depletion in *C. xerampelina* is a strong possibility because we observed males not only participating in multiple spawnings per night but also forsaking breeding opportunities to forage (unpublished data). Finally, it is possible that a percentage of males in the study population suffer intrinsically low fertility (Morrow et al. 2002). Among vertebrates, low male fertility can be attributed to a variety of causes such as disease, testicular injury and genetic disorders (Charlesworth & Charlesworth 1987; Achermann & Jameson 1999; Morrow et al. 2002). However, such problems are usually restricted to populations that have been subjected to environmental pollution (Mohallem et al. 2005) or have passed through severe genetic bottlenecks (Morrow et al. 2002). Clearly, further research is required to discriminate between the alternative explanations for variable fertilization success in *C. xerampelina*.

Irrespective of the reason(s) for variance in male fertilization capacity, our finding that group spawning enhances fertilization success is important because it provides the first evidence that this direct benefit might selectively favour polyandry in an anuran amphibian. The next question of interest is whether fertilization insurance may account for simultaneous polyandry in other frog species. Simultaneous polyandry has been reported in at least four other foam-nesting rhacophorids (*Polypedates leucomystax*, *Chiromantis rufescens*, *Rhacophorus arboreus*, *R. schlegelii*: Coe 1967; Feng & Narins 1991; Fukuyama 1991; Kusano et al. 1991; Kasuya et al. 1996) and two foam-nesting leptodactylids (*Leptodactylus chaquensis*, *L. podicipinus*; Prado & Haddad 2003). In *R. schlegelii* and *L. podicipinus*, males physically interact during spawning, so it is possible that physical interference may reduce fertilization success (e.g. *C. georgiana*: Byrne & Roberts 1999). However, in the other foam nest builders where simultaneous polyandry has been reported, levels of male–male interaction during spawning are negligible, so there is good reason to expect that females benefit directly through fertilization insurance. Empirical evaluation of the fertilization insurance hypothesis in a variety of foam-nesting frogs would provide a valuable next step towards understanding the evolution of polyandry in anuran amphibians.

To date, evidence to support the fertilization insurance hypothesis among external fertilizers has remained limited to a few species of fish and broadcast-spawning invertebrates (Petersen 1991; Levitan 2005; Smith & Reichard 2005). Our study is the first to provide evidence that fertilization insurance is a benefit of polyandry in frogs. This is an important advance because it suggests that fertilization insurance may have evolved repeatedly and independently, and might be a widespread mechanism favouring the evolution of polyandry among animals that use the external mode of fertilization.

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