



RESEARCH PAPER

Sequential Polygyny During Egg Attendance is Rare in a Tree Frog and Does not Increase Male Fitness

Wei-Chun Cheng*, Yi-Huey Chen†, Hon-Tsen Yu‡, J. Dale Roberts§,¶ & Yeong-Choy Kam*

* Department of Life Science, Tunghai University, Taichung, Taiwan

† Department of Life Science, Chinese Culture University, Taipei, Taiwan

‡ Institute of Zoology and Department of Life Science, National Taiwan University, Taipei, Taiwan

§ Centre for Evolutionary Biology, School of Animal Biology M092, University of Western Australia, Crawley, WA, Australia

¶ Centre of Excellence in Natural Resource Management, University of Western Australia, Crawley, WA, Australia

Correspondence

Yeong-Choy Kam, Department of Life Science, Tunghai University, Taichung, Taiwan 40704, R.O.C.

E-mail: biyckam@thu.edu.tw

Hon-Tsen Yu, Institute of Zoology and Department of Life Science, National Taiwan University, Taipei, Taiwan 10617, R.O.C.
E-mail: ayu@ntu.edu.tw

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Abstract

Sequential polygyny is a reproductive strategy that allows males to continue to mate and compensates for the loss of future breeding opportunities incurred by parental care (i.e. egg attendance). Using the frog *Kurixalus eiffengeri*, we tested predictions that (1) attending males fathered two, overlapping clutches; and (2) that double clutching leads to improved offspring numbers. Using five microsatellite DNA markers, we genotyped 15 pairs of overlapping clutches, which differed slightly in developmental stage at a single egg-laying site. Parentage analyses showed at least 12 of 15 pairs of overlapping egg clutches were sired by the attending male mated with different females, providing the first genetic evidence to support an earlier prediction that attending males sired both egg clutches. Field surveys found a low incidence of overlapping clutches (4.9% of 263 egg-occupied stumps), suggesting sequential polygyny is uncommon. Stumps with multiple clutches contained significantly more eggs than stumps with single clutches but hatched similar number of tadpoles. Results suggest that continuous calling that attracts females during egg attendance is a reproductive tactic that maximizes mating opportunities. However, adoption of the sequential polygyny tactic may only result in marginal fitness gains for males that are traded off against average higher egg mortality in larger egg clutches.

Parental investment is any parental effort put into an individual offspring that increases the offspring's survivorship and hence the parent's reproductive success. However, parental investment is at the cost of the parent's ability to invest in other offspring and consequently future reproductive success (Williams 1966; Trivers 1972; Sargent & Gross 1986). Time and the energy expended in gamete production and parental care are two major components of parental investment. As most male animals devote less time and energy to gamete production than females, reproductive success for males is usually determined by the number of successful matings rather than gamete production (Trivers 1972; Krebs & Davies 1993; Shuster & Wade 2003).

Males can benefit more than females from increasing the number of mates, and therefore, male parental care is not expected if the time or energy allocated to current offspring results in a decrease in future mating opportunities (Sargent & Gross 1986; Krebs & Davies 1993; Manica & Johnstone 2004; Alonzo 2012). The reproductive strategies adopted by males are therefore shaped by the costs and benefit of parental care, that is, the trade-off between current and future reproductive outputs (Trivers 1972; Clutton-Brock 1991).

Paternal egg attendance, a male parent remaining with an egg mass at a fixed location, is a common form of parental care in oviparous animals (Crump 1995; Smith & Wootton 1995; Manica & Johnstone

2004; Wells 2007). Care behavior includes egg attending, egg brooding and fanning by fishes and egg guarding, turning, brooding, and oophagy to remove infected eggs by terrestrial and arboreal frogs (Crump 1995; Smith & Wootton 1995). Although egg attendance has been documented to reduce the mortality of eggs and in turn increase the current reproductive success of male parents, it also incurs a cost that includes greater risk of predation, less opportunity to forage (less energy gain), and fewer or complete loss of mating opportunities (Gross & Sargent 1985; Clutton-Brock 1991; Crump 1995). Attending male fishes can obtain several egg clutches in a nest (Wootton 1984; Smith & Wootton 1995), and similar findings have been reported in frogs where males who call continuously during egg attendance can obtain additional mates (i.e. sequential polygyny) (Woodruff 1977; Junca 1996; Burrowes 2000; Vockenhuber et al. 2008), resulting in overlapping egg clutches with slight differences in developmental stage. The occurrence of overlapping egg clutches in a nest may be a reproductive strategy used by attending males to compensate for loss of mating opportunities while attending eggs (Woodruff 1977; Burrowes 2000). However, there are two key assumptions that need to be verified before discussing the ecological and evolutionary significance of this reproductive tactic: (1) are both clutches fathered by the attending male? (2) does caring for two clutches lead to enhanced fitness (measured by the number of offspring)? It is often assumed that the overlapping clutches are sired by one male; however, the paternity patterns are not always obvious due to difficulties in making appropriate field observation and/or use of undetected alternative mating tactics (Petrie & Kempnaers 1998; Avise et al. 2002; Zheng et al. 2010; Roberts & Byrne 2011). For example, social monogamy in birds has repeatedly been shown to be associated with extra-pair mating (Lifjeld et al. 1993; Petrie & Kempnaers 1998). Furthermore, addition of an egg clutch inevitably increases the number of eggs in a nest; however, this does not necessarily yield more offspring. In aquatic animals, embryonic oxygen consumption increases with the number of eggs in a nest, and male fish need to fan more to increase oxygen levels in the water (Reebs et al. 1984; Torricelli et al. 1985; Coleman & Fischer 1991). Not all attending males may be able to increase fanning for effective aeration of very large clutches. In insects, large egg clutches may attract predators or parasitoids because of high egg detect-

ability (Kaireva & Odell 1987; Dobson 1988) or indirectly by volatile emissions from host plants (Hoballah & Turlings 2001; Kessler & Baldwin 2001).

In this study, we combined field observations and molecular parentage analyses to evaluate the reproductive strategies used by egg-attending male *Kurixalus eiffingeri* (formerly known as *Chirixalus eiffingeri*). *Kurixalus eiffingeri* deposits eggs on hard surfaces above the water line in bamboo stumps or tree hollows. During the breeding season (February to August), males occupy stumps with standing water and exhibit territorial behavior. Territorial male frogs call at the opening of bamboo stumps to attract females. Most matings involve one male, but parentage analyses using microsatellite DNA markers showed that about 16% of egg clutches were produced by simultaneous polyandry (Chen et al. 2011). Eggs are attended for 9–14 days by a male, even when there is more than one father, until the tadpoles hatch (Kam et al. 1998a). Upon hatching, tadpoles drop into the pool of water where they grow and develop until metamorphosis. Tadpoles are obligatorily oophagous and are fed by females that lay unfertilized, trophic eggs directly in the water, in the absence of male frogs (Ueda 1986; Kam & Yang 2002). During the embryonic period, male frogs actively moisten egg clutches using their ventral surface, presumably to prevent desiccation of egg clutches (Ueda 1986; Chen et al. 2007). Care effort is correlated with paternity share of attending males (Chen et al. 2011). About 6% of egg-attending males have been observed calling at the opening of bamboo stumps (Chen 2006), and overlapping clutches have been reported (Cheng & Kam 2010), suggesting a strong possibility of the occurrence of sequential polygyny as a reproductive tactic.

Specifically, we investigated (1) the paternity contribution of the attending male to the two egg clutches in a stump; (2) the prevalence of multiple clutches in bamboo stumps; or (3) the impact of total egg number on overall egg survival. Earlier studies have shown that bamboo stumps are limited and vary in water holding capacity (Lin & Kam 2008; Lin et al. 2008). During the breeding season, males occupy stumps with standing water and defend nest sites against intruders (Chen et al. 2011). Given that bamboo stumps are a spatially discrete microhabitat that can easily be guarded against entry by other males, we predicted that the attending male would call and attract additional female frogs resulting in multiple clutches. In addition, we predicted that the occurrence of multiple clutches is common in *K. eiffingeri*,

and nests with multiple clutches have larger total clutch size and hatch more tadpoles than stumps with single clutches.

Materials and Methods

Study site

We conducted behavioral observations on male frogs in the bamboo forests of the Experimental Forest of National Taiwan University at Chitou (elevation 1016 m, approximately 23°39'20"N, 120°48'10"E) in Nantou County, Taiwan. The mean annual temperature at Chitou is 18°C with approximately 3000 mm of rainfall annually (Kam et al. 1996). The bamboo, *Phyllostachys edulis*, was the most abundant vascular plant species at the study site. The bamboos are cut periodically for commercial purposes, and the stumps remain for several years before collapsing. Bamboo stumps collect rain water and become oviposition sites and tadpole development sites for *K. eiffingeri* (Kam et al. 1996).

Field study of the prevalence of overlapping clutches

From April through August 2003, 1–3 times per week during daytime in several bamboo forests, we located egg clutches of *K. eiffingeri* by intensive surveys of bamboo stumps containing standing water. Once an egg clutch was found, we recorded the clutch size and developmental stage of embryos (Gosner 1960). Embryonic development and conditions can be monitored with the naked eye, and embryos after Gosner stage 18 responded to our gentle tapping on the bamboo stumps. After the initial measurements, we monitored the number and development of eggs. An increase of egg number in a stump indicated a new egg clutch had been laid, that is, multiple clutches were present. The eggs of second clutches were usually deposited in the same area inside the bamboo as earlier clutches, but difference in the developmental stages of eggs permitted identification of individual clutches (Fig. 1). Observations were discontinued when the eggs hatched or died.

Sample collection for parentage analyses and ethical consideration

For parentage analyses, we collected tissues from attending males and tadpoles from overlapping clutches (≥ 10 tadpoles/clutch for discrete clutches which can be separated by developmental stage or 20



Fig. 1: Two egg clutches of *Kurixalus eiffingeri* in two different developmental stages seen laid contiguously on the inner wall above water line inside a bamboo stump. The white arrow indicates the earlier clutch (~Gosner stage 17), and the striated arrow indicates the later clutch (~Gosner stage 10).

tadpoles if the two clutches were mixed, and clutch identity could not be defined by developmental stage or position). In 2003, we obtained 13 overlapping clutches from the field investigation on the prevalence of overlapping clutches described above. Eggs and hatched tadpoles grew and developed in the field, and we collected tadpole samples after they reached around Gosner stage 40. Due to high tadpole mortality in the field (Kam et al. 1998b; Chen et al. 2001), we only successfully collected enough tadpole samples from two stumps (stump # S1 and S2) for parentage analyses. To increase sample sizes, we collected additional 13 samples in May–August of 2007–2009. In these 3 yrs, we did not study the prevalence of overlapping clutches but collected samples solely for parentage analyses. In general, we monitored stumps 1–2 times a week in one to two bamboo forests and marked egg-occupied stumps. If we noticed an increase in the number of eggs, indicating a second clutch, in any egg-occupied stump, we recorded the clutch size and developmental stages of embryos of each clutch, collected, and transported them back to the laboratory. We then separated the two egg clutches by developmental stage and incubated each clutch separately on moist substrate until hatching. For stumps S4–5, eggs from two clutches (detected by an increase in egg number) could not be separated by developmental stage, and so we incubated the two clutches together until hatching. In the laboratory, tadpoles were raised in beakers (ca. 1 L water) and fed with raw chicken egg yolk once every 4 days until

they reached metamorphosis. An earlier study showed that chicken egg yolk is a good substitute for *K. eiffingeri* eggs, and tadpoles that are fed once every 4 days grow as well as tadpoles that were fed by a female frog (Liang et al. 2002).

We toe-clipped attending male frogs and preserved tissues individually in 95% ethanol. We sterilized scissors with alcohol, clipped only the most distal segment of the second toe, and applied antibiotic cream to the wound. Based on four previously published studies, McCarthy & Parris (2004) concluded that toe-clipping reduced the return rate by 4–11% for each toe removed after the first toe. Because we removed only a distal segment of a toe for parentage analyses, the impact of toe-clipping on individuals and on the population as a whole is likely to be substantially less than in studies in which two or more toes are removed (Crafe et al. 2011).

In all years when tadpoles reached Gosner stage 40, we tail-clipped tadpoles and preserved tissues in 95% ethanol for parentage analyses. We used a plastic spoon to scoop tadpoles from the water and scissors, sterilized by alcohol, to cut the distal portion of the tail (i.e. 10% or less of total tail). The tissue sampling procedure took about 2 min. The tip of the tadpole tail is less vascularized than other areas. Consequently, most tadpoles did not bleed after clipping and swam immediately after we put them back in the water. Wounds healed 1–2 days after clipping. *Kurixalus eiffingeri* tadpoles are sedentary and remain immobile most of the time (Kam & Yang 2002), and tail-clipping has minimal effects on the activity and feeding of tadpoles. In 2003, we followed the growth and survival of tail-clipped tadpoles in a bamboo forest once a week until they reached metamorphosis and left the bamboo stumps (at about Gosner stage 43–45). One week after tail-clipping, only 2.03% (12 of 591) tadpoles died, and most tadpoles survived and reached metamorphosis (Y.-H. Chen, unpubl. data). When tadpoles reached metamorphosis, froglets were released back to the bamboo forests. Throughout the study period, we adhered to the guidelines for the use of animals in research and the legal requirements of the Tunghai University and National Science Council of Taiwan in conducting these experiments.

Genotyping

The DNA extraction, amplification, and genotyping of five microsatellite DNA markers were described by Chen et al. (2011). Briefly, genomic DNA was extracted from each sample using Classica™ Genomic DNA iso-

lation kits (Lamda Biotech, Inc., St. Louis, MO, USA), and five polymorphic microsatellite loci (*CEd12365*, *CEd15688*, *CEd19063*, *CEd13854*, and *CEd19091* with the forward or reverse primer being end-labeled with HEX, FAM, or TAMRA fluorescent dye) were amplified using PCR. The PCR of each locus was run individually, and the PCR products from different dye-labeled loci were pooled before capillary *electrophoresis*. The sizes of the microsatellite alleles were scored with GENETIC PROFILER 2.2 software (Amersham, GE, Piscataway, NJ, USA). Genotyping errors for the respective loci were 1.2, 2.5, 2.5, 0, and 0%.

Parentage Analyses

Chen et al. (2011) reported allele numbers, allele frequencies, null allele frequencies, and exclusion probabilities for each locus using the program CERVUS 3.0 (Field Genetics Ltd., London, UK) (Marshall et al. 1998; Kalinowski et al. 2007). Simulations in GENEPOP (Raymond & Rousset 1995) for the frog samples revealed no linkage disequilibrium ($p > 0.05$). The combined exclusion probability across all loci was 0.987 when the genotype of a parent is known.

We used a likelihood-based COLONY 2 program (Wang 2004) and an exclusion–deduction method (assuming Mendelian inheritance rules) (Danzmann 1997; Myers & Zamudio 2004) to analyze genetic relationship between the attending males and the multiple clutches in the nests and maternity of overlapping clutches. COLONY 2 implements a maximum likelihood method to partition the sampled offspring into full-sib families nested within half-sib families (Wang 2004). It provides the most probable configuration of paternity, including assignment of every offspring to one of the candidate or estimated parental genotypes (Jones et al. 2010). This program can also detect and correct potential effects of genotyping errors—appropriate for our analyses given we have a low rate of genotyping error. Criteria setting for parentage analyses was described by Chen et al. (2011).

In the exclusion–deduction method, we first used PROBMAX 1.3 (<http://www.uoguelph.ca/~rdanzman/software.htm>) (Danzmann 1997) to analyze the genetic compatibility between attending males and tadpoles in two clutches. We allowed one mismatch to minimize false parentage exclusion. We then used a deduction method that was similar to the minimum method of Myers & Zamudio (2004) to deduce the parentage relationship. This method inferred a minimum number of parents in a clutch based on allele counts. An increase of two alleles represented one more adult contributing to the clutch. In cases where

the attending male was the genetic father of the entire clutch, the minimum number of mothers for the clutch can be estimated by counting the non-paternal alleles. If one putative mother was indicated by two (or less) non-paternal alleles, genotype of the putative mother can be deduced by subtraction. Whether the overlapping clutch belonged to the same mother can be determined by simply comparing multi-locus genotype of the putative mothers. In cases where the attending male was the genetic father of part of the total clutch, we divided the clutch into two sub-groups (i.e. genetically related and unrelated) and repeated the procedure to determine parentage separately.

Statistical analyses

All statistical analyses of the data were performed with SAS (SAS Institute Inc. 2001). We used the Wilcoxon rank-sum test to compare the clutch size and number of tadpoles between single and multiple clutches. Unless stated otherwise, we reported the mean ± SD of each variable.

Results

Frequency and characteristics of overlapping egg clutches

In 2003, we found 263 stumps containing egg clutches with an average of 59.4 ± 27.5 eggs/clutch. Developmental stage within clutches was consistent, but stage varied between clutches depending on the time between egg deposition and collection. We detected an increase of egg number in 13 stumps (4.9% of total stumps), indicating a new clutch was added. Average number of tadpoles hatched per stump was 11.2 ± 13.7 (range 0–76, coefficient of variation = 122%; n = 263 stumps). Mean hatching rate was $19.2 \pm 21.7\%$ (range = 0–100, coefficient of variation = 113%; n = 263 stumps).

Unsurprisingly, stumps with multiple clutches (104.5 ± 25.3 , n = 13) had significantly more eggs than those with a single clutch (57.1 ± 25.6 , n = 250; Wilcoxon rank-sum test: $Z = 4.954$, $p < 0.0001$; Fig. 2). However, both stump types hatched similar number of tadpoles (multiple-clutch stumps, 9.1 ± 11.2 ; single-clutch stumps, 11.4 ± 13.9 , $Z = 0.512$, $p = 0.598$; Fig. 2).

Parentage analyses

We collected a total of 529 tadpoles from overlapping clutches for parentage analyses: 2003 (2

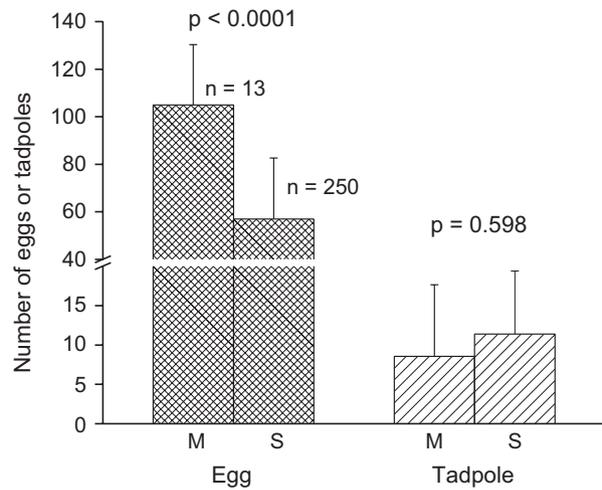


Fig. 2: Number (means ± SD) of eggs (cross-hatched) and tadpoles (striated) in multiple (denoted by M, n = 13) and single (denoted by S, n = 250) clutches.

Table 1: Summary of parentage analyses of overlapped egg clutches by the COLONY program and an exclusion-deduction method

	COLONY	Exclusion-deduction method
Number of stumps (out of 15) where the attending male was the father (sole or partial) of both clutches	14	12
Number of stumps (out of 10) where overlapped egg clutches were produced partially or completely by different females ^a	9	9

^aTadpoles of egg clutches in five stumps (i.e. S1–S5) were mixed during collection. Parentage analyses showed that there were at least two mothers, but the mating patterns could not be determined, thus, these data were excluded from maternity analysis.

clutches), 2007 (5 clutches), 2008 (1 clutch), and 2009 (7 clutches). COLONY and exclusion-deduction methods deduced that overlapping egg clutches in 14 and 12 of 15 stumps, respectively, were partially or completely sired by the attending male, but egg clutches in one stump (i.e. stump S13) were sired by different males (the first egg clutch was sired by the attending male, the second clutch was sired by two unknown males) (Table 1). The discrepancy in parentage analyses between the two methods was in stumps S1 and S9 where COLONY deduced egg clutches were partially or completely sired by the attending male, but the exclusion-deduction method deduced eggs clutches were sired separately by unknown males with or without the attending male (Appendix 1).

In contrast, the majority of overlapped egg clutches were produced by different females. COLONY and the exclusion–deduction method both deduced that overlapping egg clutches in 9 of 10 stumps egg clutches were produced by different females (Table 1), but in one stump (i.e. stump S10) the first egg ‘clutch’ was produced by two females, and the second egg clutch was produced by one of the earlier females (Appendix 1).

Throughout our study, we collected overlapped egg clutches in 10 stumps (Stump 6–15) that we were able to categorize into early- and late-laid clutches. We assumed each egg clutch (i.e. a total of 20 egg clutches; Appendix 1) was the product of a single mating event. Based on parentage analyses using COLONY, we indeed found that 16 of 20 egg clutches were each produced by a single mating event, but 4 egg clutches (i.e. an early-laid clutch in stump S10 and three late-laid clutches each in stump S8, S13, and S15) had two mothers in an egg ‘clutch’, indicating each egg ‘clutch’ was produced by two mating events that occurred very close together in time resulting in no detectable difference in developmental stage. Further analyses of these 24 egg clutches (16 single clutches + 4 double clutches each produced by two mating events) revealed a high occurrence of multiple paternity ($9/24 = 37.5\%$) with two to three fathers in each event.

Discussion

Parentage analyses showed that most overlapping egg clutches in a stump were a result of sequential breeding (or polygyny) of the attending male, providing the first genetic evidence to support earlier predictions that attending anuran males can sire and care for multiple egg clutches in a single egg deposition site (Woodruff 1977; Junca 1996; Burrowes 2000; Vockenhuber et al. 2008). Parental care is a substantial expenditure of energy and time, and the trade-off between cost and benefit influences the pattern of parental care and reproduction (Williams 1966; Trivers 1972; Sargent & Gross 1986; Manica & Johnstone 2004). Reduced or lost mating opportunities are costs for males with parental care, and males are expected to adopt strategies to avoid or reduce this cost (Sargent & Gross 1986; Clutton-Brock 1991). Calling to attract mates during egg attendance is an alternative reproductive strategy to compensate for the loss of future mating opportunities (Townsend 1986, 1989a; Burrowes 2000; Alonzo 2012). If *K. eiffingeri* males can attend eggs without losing mating opportunities, then they could gain a twofold benefit by (1) increas-

ing the survival of the current offspring by male attendance; and (2) obtaining extra access to fertile females (Clutton-Brock 1991; Burrowes 2000; Cezilly et al. 2000).

The field results demonstrated a low occurrence of multiple clutches probably as a result of the infrequent calling activities of attending male *K. eiffingeri* (only 6% of attending males called) (Chen 2006). Similar results were found in studies with other frog species: male *Eleutherodactylus coqui*, *Centrolenella fleischmanni*, *Mantidactylus bicalcaratus*, and *M. punctatus* all reduced or stopped calling during egg attendance (Townsend et al. 1984; Jacobson 1985; Townsend 1986; Hayes 1991; Lehtinen 2003). In *E. coqui*, male frogs call only in elevated sites, and the ground-nesting males cannot call without leaving their clutch unprotected. Leaving the clutch temporarily can lead to heavy predation on offspring, and this may outweigh the benefit of obtaining additional matings (Townsend 1986, 1989b). In this study, the cost of obtaining additional mating opportunities is low because attending *K. eiffingeri* males called at the opening of stumps and could obtain additional mates without leaving the eggs unattended. However, the male-biased operational sex ratio (Lin et al. 2008) and extended maternal care (egg provisioning to tadpoles for about 1–2 month) that prevents females from breeding (Kam et al. 1998a) may discourage males from calling while attending eggs (Sargent & Gross 1986): Both attributes reduce female availability (Manica & Johnstone 2004).

More importantly, attending male *K. eiffingeri* did not gain any benefit from adding a second egg clutch to a nest because larger egg clutches did not yield more tadpoles. In assassin bugs, larger egg clutches also lead to higher rates of parasitism and/or cannibalism (Gilbert & Manica 2009), but the impacts on other species with sequential polygyny are not documented. This is an important deficiency as in earlier studies on sequential polygyny in frogs—increases in fitness are assumed or implied but not demonstrated. It is also worthwhile noting that bamboo forests in this study probably increase frog densities over natural breeding sites. Unnaturally, high breeding densities may result in reduced fitness through the so-called ecological traps (Schlaepfer et al. 2002) where animals are attracted to and use inappropriate resources because they exhibit cues normally associated with reproductive success. We have no data on reproductive performance in natural breeding sites so can make no further comment but recognize this as possibly contributing to our results.

Desiccation, predation, and fungal infection are major causes of egg mortality in *K. eiffingeri* (Kam et al. 1996; Chen et al. 2007; Cheng & Kam 2010). Larger egg clutches in *K. eiffingeri* may increase egg detectability by predators such as slugs and ants and increase the likelihood of fungal infection (Kam et al. 1997). High humidity in the bamboo stump may favor fungal growth, and the larger egg clutches may be more susceptible to fungal infection as the proximity of eggs allows rapid spread of fungal hyphae (Kam et al. 1996, 1997).

For sequential polygyny to have a fitness advantage, there must be something other than a simple numerical advantage due to increased egg number. We consider two possibilities: paternity of attending males and benefits of maternal provisioning of hatched tadpoles. In this study, rates of simultaneous polyandry are double those reported previously by Chen et al. (2011): in 4 of 10 stumps where the first egg clutch (Stump S07, S11, S12, and S14) was produced by simultaneous polyandry, resulting in multiple paternity. In *K. eiffingeri*, egg clutches are attended by one male (Chen et al. 2007), and thus, in the case of simultaneous polyandry, the uncertainty of paternity may encourage the attending male to breed again while still attending eggs to maximize paternity share. On the other hand, when a male mates with two females sequentially, he may gain benefits by having two females to feed his offspring during the larval period. Tadpoles are obligatorily oophagous, and their only food source is unfertilized trophic eggs laid by females (Kam et al. 1996, 2000). Kam et al. (1998a) demonstrated that availability of trophic eggs is limited; thus, tadpoles would grow faster if both females return to feed or at least be fed if one female does not return.

In conclusion, molecular evidence showed that overlapping clutches were sired by attending males, suggesting *K. eiffingeri* males may use sequential polygyny as a reproductive strategy to compensate for the loss of mating opportunities while attending eggs. The cost of obtaining additional mates by *K. eiffingeri* males may be low; however, male-biased operational sex ratios and the long period of maternal care, together with the fact that larger egg clutches by sequential polygyny did not yield more offspring due to the clutch size-dependent mortality of eggs may explain why this reproductive tactic is uncommon in this species.

We contend that cost–benefit analyses on both mate acquisition and the fate of offspring are needed to completely understand the ecological and evolutionary significance of sequential polygyny

involving parental care. It is likely that ecological constraints, such as the distribution and abundance of mates, availability of breeding sites, predators, and conditions of breeding sites may all play critical roles in determining how frequently this tactic will be used.

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Appendix 1: Summaries of stump use, clutch size, development stage, sample size used, and results of parentage of offspring and attending males deduced from the COLONY program and an exclusion-deduction method.

Year	Stump#	Egg # of early (1) and late (2) clutches	Gosner stages	Sample size	Mating pattern (COLONY) ^a	Mating pattern (Exclusion-deduction method) ^a	
2003	S01 ^b	1 = 77 2 = 33	23, 17	30	♂A♀1–2	2♂2♀not including♂A	
	S02 ^b	1 = 66 2 = 45	11, 3	22	♂A♀1–4	♂A♀1–4	
2007	S03 ^c	1 = 52 2 = 81	17 14–15	40	♂A,1♀1 + ♂A♀2	♂A♀1–3	
	S04 ^d	1 = 47 2 = 42	8–9 8–9	40	♂A♀1–2	♂A♀1–2	
	S05 ^d	1 + 2 = 103	14–15	27	♂A♀1–2	♂A♀1–2	
2008	S06	1 = 58 2 = 40	14–15 7–8	21 19	♂A♀1 ♂A♀2	♂A♀1 ♂A♀2	
		S07	1 = 29 2 = 40	20 17	20 20	*♂A,1–2♀1 *♂A,1♀2	♂A♀1 + ♂1♀1–2 ♂A,2♀3
	S08	1 = 44 2 = 40	21–22 17	22 18	♂A♀1 *♂A,1♀2 + ♂1♀3	♂A,1♀1 2♂2♀including♂A	
		S09	1 = 33 2 = 66	20 12–13	10 20	♂A♀1 *♂A,1♀2	♂1♀1 ♂A,2–3♀2 + ♂4♀3
	S10		1 = 13 2 = 43	25 18	20 19	♂A♀1 + ♂A♀2 ♂A♀1	♂A♀1 + ♂A♀2 ♂A♀1
			S11	1 = 36 2 = 71	17 10	20 20	*♂A,1♀1 *♂A,2♀2
	S12	1 = 29 2 = 31		20–21 12	20 19	*♂A,1♀1 ♂A♀2	♂A,1♀1 ♂A♀2 + ♂A♀3
S13		1 = 27 2 = 38	25–26 12	15 20	♂A♀1 ♂1♀2 + ♂2♀3	♂A♀1 3 different parents	
	S14	1 = 47 2 = 42	19–20 16	20 17	*♂A,1♀1 *♂A,2♀2	♂A♀1 ♂A♀2	
S15		1 = 11 2 = 52	21–22 17	10 20	♂A♀1 ♂A♀2 + ♂A♀3	♂A♀1 ♂A♀2	

Note ^a♂A is attending male, ♂x, and ♀y are male x and y, respectively. For example, ♂A,1♀1 + ♂A♀2 represents an “egg clutch” was produced by two mating events that included mating event one (attending male, male 1 and female 1) and two (attending male and female 2). Tadpoles of egg clutches in five stumps (i.e. S1–S5) were mixed during collection. Parentage analyses showed that there were at least two mothers (e.g., ♂A♀1–2 represents an overlapping egg clutch was produced by one father (i.e. attending male) and two mothers), but the mating patterns could not be determined.

^bWe did not collect eggs of two overlapped clutches until tadpoles hatched and were mixed.

^cWhen we found overlapped clutches in stump S03, the developmental stages of embryos of stump were quite different. Due to a delay of field schedule, the embryos of two clutches were difficult to separate by the time we collected them.

^dEmbryos of two clutches in stump S04 and S05 had similar developmental stage at the time of collection, thus, we collected and mixed them.

*Parentage analyses of egg clutch showed multiple paternity which was a result of simultaneous polyandry.