

# **PARENTAGE OF OVERLAPPING OFFSPRING OF AN ARBOREAL-BREEDING FROG WITH NO NEST DEFENSE: IMPLICATIONS FOR NEST SITE SELECTION AND REPRODUCTIVE STRATEGY**

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## **Abstract**

Overlapping offspring occurs when eggs are laid in a nest containing offspring from earlier reproduction. To unveil the parentage between overlapping offspring and parents is critical in understanding oviposition site selection and the reproductive strategies of parents. Amplectant pairs of an arboreal-breeding frog, *Kurixalus eiffingeri*, lay eggs in tadpole-occupied nests where offspring of different life stages (embryos and tadpoles) coexist. We used five microsatellite DNA markers to assess the parentage between parents and overlapping offspring. Results showed varied parentage patterns, which may differ from the phenomenon of overlapping egg clutches reported earlier. Parentage analyses showed that only 58 and 25% of the tadpole-occupied stumps were reused by the same male and female respectively, partially confirming our prediction. Re-nesting by the same individual was more common in males than females, which is most likely related to the cost of tadpole feeding and/or feeding schemes of females. On the other hand, results of parentage analyses showed that about 42 and 75 % of male and female respectively bred in tadpole-occupied stumps where tadpoles were genetically unrelated. Results of a nest-choice experiment

revealed that 40% of frogs chose tadpole-occupied bamboo cups when we presented identical stumps, without or with tadpoles, suggesting that the habitat saturation hypothesis does not fully explain why frogs used the tadpole-occupied stumps. Several possible benefits of overlapping offspring with different life stages were proposed. Our study highlights the importance of integrating molecular data with field observations to better understand the reproductive biology and nest site selection of anuran amphibians.

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**Keywords:** Amphibian, nest-site selection, overlapping offspring, parentage

## **Introduction**

Overlapping offspring is an interesting phenomenon that occurs more commonly than originally thought, but the patterns, causes, and ecological and evolutionary consequences are not fully understood. When sites are reused for reproduction, the sites may be empty (if previous offspring have left) or may contain offspring from earlier reproduction. If offspring from previous reproduction remain, this results in two overlapping cohorts.

The parentage between overlapping offspring and nest users could lead to differences in reproductive strategy and nest site selection. When an adult reuses a nest occupied by a conspecific, it may contain genetically related or unrelated offspring. The former could be a case of nest-site fidelity (Hoover, 2003; Vergara, Aguirre, Fargallo, & Davila, 2006) whereas the latter could be a case of oviposition site selection could be a case of the saturation of breeding sites that force breeding pairs to oviposit in an occupied site (Doody, Freedberg, & Keogh, 2009) or of conspecific attraction because the presence of conspecifics may represent the quality of breeding resources (Doligez, Danchin, & Clobert, 2002; Mokany & Shine, 2003; Rudolf & Rodel, 2005).

In this study, we used a Taiwanese frog (*Kurixalus eiffingeri* (Anura: Rhacophoridae)) that breeds in water-filled bamboo stumps as a model animal to study the parentage between overlapping offspring and its ecological consequence on reproductive strategy and nest site selection. Specifically, we used (1) five microsatellite DNA markers to analyze the parentage of adults and tadpoles and (2) paired bamboo cups with and without tadpoles to study the nest choice of frogs and to reveal the possible causes of nest reuse.

## **Materials and methods**

We conducted experiments in the bamboo forests at Chitou in Nantou County, Taiwan. From March – August 2007-2009, we conducted field surveys to collect adults (males and/or females) and overlapping offspring

(eggs and tadpoles) for parentage analyses. We captured and toe-clipped attending male and feeding female frogs and preserved the tissue individually in 95% ethanol.

In the laboratory, we incubated egg clutches separately on moist substrates until hatching. We reared tadpoles in beakers (ca. 1 L water) and fed them with chicken egg yolk once every 4 days until they reached metamorphosis. When tadpoles reached Gosner stage 40, we clipped a distal portion of tadpoles' tail (i.e., 10% or less of total tail length) and preserved tissues in 95% ethanol for parentage analyses.

We conducted genetic analyses on the parentage of tadpoles and adults. Detailed methods on parentage analyses were reported by Chen et al (Y.-H Chen, Cheng, Yu, & Kam, 2011). The likelihood-based, COLONY 2 program (Wang, 2004) was used to analyze genetic relationships between the attending males, feeding females and the offspring in the nests. Based on the results of the parentage analyses, we deduced the mating pattern of frogs and further assessed whether individuals that breed earlier in a stump would reuse the same stump again.

From April 17 to August 15, 2013, we also conducted a manipulated experiment where paired bamboo cups, with and without tadpoles, were set up to investigate nest choice of frogs. We randomly designated one cup as the control and the other as the experimental group. A control group contained water only but the experimental group contained water and 5 tadpoles (Gosner stage 28-35). We surveyed the paired-bamboo cups every 4 days to determine which cup was oviposited.

## **Results and discussion**

COLONY deduced eight different types of parentage among overlapping offspring. The most common parentage among overlapping offspring was partially the same father and different mothers ( $N = 8$ ), followed by different parents ( $N = 7$ ). The former is a case when a male frog was involved in matings that sired offspring in the early- and late-laid clutches, and at least one of the clutches was either synchronous polyandry or sequential multi-mating events that included other male frogs, which resulted in multiple paternity. In contrast, the latter is a rather simple case where two different pairs of frogs produced the two cohorts of offspring. The remaining types of parentage among overlapping offspring occurred at much lower frequency: same male and female ( $N = 1$ ), same male and partially the same female ( $N = 1$ ), same male and different female ( $N = 3$ ), partially the same male and female ( $N = 1$ ), different male and same female ( $N = 2$ ), and different male and partially the same female ( $N = 1$ ).

Based on results of parentage among overlapping clutches, we found that a total of 14 stumps were reused by the same male frogs but 10 stumps

were not, and the proportion of stump use is statistically similar (G test,  $G = 0.670$ ,  $P = 0.413$ ). On the other hand, a total of 6 stumps were reused by the same female frogs but 18 stumps were not, and the proportion of stump use was statistically different (G test,  $G = 6.279$ ,  $P = 0.012$ ).

We conducted 28 surveys, checking a total of 1862 cup pairs from April to August and found that 57 egg clutches were deposited during the study period (Table 2). Most egg clutches were found between May to July when the breeding activity peaked. A total of 34 and 23 egg clutches were laid in control and experimental bamboo cups, respectively, and the egg placement was independent from cup treatment (G test,  $G = 2.136$ ,  $P = 0.144$ ). During this three month period (May to July), 40, 31, and 52% of egg clutches were laid in the experimental cups in the respective months. Clutch size of eggs laid in control cups ( $45.3 \pm 18.4$  eggs,  $N = 24$ ) was not different from that of experimental cups ( $42.8 \pm 19.4$  eggs,  $N = 17$ ; Wilcoxon rank sum test,  $W = 229.5$ ,  $P = 0.508$ ).

The casual mechanism(s) of the varied patterns of parentage in overlapping offspring in this study are yet to be clarified and are expected to be more complicated than that of overlapping egg clutches reported earlier. We speculate that the varied parentage patterns can at least be associated with two ecological factors: a lack of nest defense and limited breeding resources. *Kurixalus eiffingeri* has a unique “sequential” form of parental care: male frogs guard eggs during embryonic period, and female frogs feed tadpoles alone during larval period; however, there is no evidence of nest or mate defense. Nest defense is a common parental behavior among insects (Gruter, Karcher, & Ratnieks, 2011), fishes (Knouft & Page, 2004), salamanders (Bachmann, 1984; Forester, 1979, 1983), frogs (Wells, 1977, 2007), and birds (Campobello & Sealy, 2011; Redmond, Murphy, Dolan, & Sexton, 2009; Westmoreland, 1989) and functions to protect valuable resources inside (e.g., mates, offspring, food, even the nest site itself (Gruter et al., 2011)). In birds, the increased frequency of extra-pair mating or paternity has been associated with a lack of territorial or mate-guarding behavior (Ewen, Armstrong, Ebert, & Hansen, 2004; Macdougall-Shackleton, Robertson, & Boag, 1996; Moller, 1990; Rowe & Weatherhead, 2007). This may also be true in *K. eiffingeri* in that a lack of mate and nest defense opens possibilities for a male or female frog to mate with others and utilize the tadpole-occupied nests to breed again. Furthermore, the quality and availability of water pools in stumps vary in time and space (Lin, Lehtinen, & Kam, 2008). As the reproductive season progresses, more and more of the arboreal pools may already have been used for breeding by other individuals (Lin et al., 2008). The competition for stumps (empty or tadpole-occupied) for breeding is heavy, which inevitably results in the reuse of

tadpole-occupied stumps. This likely leads to a diverse pattern of parentage in the overlapping offspring.

Our findings that 58 and 25 % of *K. eiffingeri* males and females respectively reused the same stump to breed partly agreed with our prediction. Earlier studies have reported site fidelity in stream-dwelling frogs which probably are able to obtain sufficient ecological necessities such that moving away from a site is not required (Daugherty & Sheldon 1982; Kam & Chen, 2000; Tessier, Slaven, & Green, 1991). In this study, the lower rate of reusing the same nest by females can probably be explained by the high cost of tadpole feeding.

On the other hand, results of parentage analyses showed that about 42 and 75 % of males and females respectively bred in tadpole-occupied stumps where tadpoles were genetically unrelated, which leads to the following question: why did frogs breed in the tadpole-occupied stumps of others? One explanation is that amplexant pairs may be forced to lay eggs in tadpole-occupied nests when most suitable breeding habitats are already used later in the breeding season (i.e., the saturated habitat hypothesis) (Doody et al., 2009). However, in the nest choice experiment, when we presented identical cups, without or with tadpoles, 40% of frogs still chose tadpole-occupied cups, suggesting that the habitat saturation hypothesis does not fully explain why frogs used the tadpole-occupied stumps. We contended that even though the quality of tadpole-occupied stumps is discounted due to inter-clutch tadpole competition, they are still as good as, if not better than, the remaining unoccupied stumps (Lin et al., 2008).

There are several possible benefits of reusing tadpole-occupied stumps. First, the presence of early-clutch tadpoles may serve as a cue for the high quality of the stumps, such as the availability and persistence of the water resource, which is particularly critical for offspring living in container habitats like tree holes and stumps which are prone to desiccation (Kitching, 2000; Rudolf & Rodel, 2005; Srivastava et al., 2004). Second, the coexistence of two cohorts of tadpoles reduces the probability of smaller tadpoles (i.e., late-laid clutches) being eaten not only because of the attack abatement effect but also because they are less conspicuous due to small size when compared to larger tadpoles (i.e., early-laid clutch) (Doody et al., 2009). Third, coexisting tadpoles in stumps could potentially be fed by two females which reduces the probability of catastrophic nest mortality. Earlier studies on *K. eiffingeri* have reported nest failure (~30%) which is due to nest abandonment by females and/or death of female frogs, mostly by snake predation (Y.-H. Chen, Su, Lin, & Kam, 2001; Chiu & Kam, 2006; Kam, Lin, Lin, & Tsai, 1998b). Since females cannot discriminate kin from non-kin (Kam, Chen, Chen, & Tsai, 2000), if a nest is used by two females (i.e.,

multiple feeders effect), the tadpoles would still be fed if one of the mothers deserts the nest or is eaten.

## Conclusion

In conclusion, integrating parentage into the discussion of nest site selection can lead to new insights into the reproductive strategies and sexual selection of animals. This is particularly true in the studies of reproductive behavior of externally fertilizing animals such as anuran amphibians and fishes because many species have complex reproductive behavior that cannot easily be detected in the field (Avise et al., 2002; Cheng, Chen, Yu, Roberts, & Kam, 2013; DeWoody & Avise, 2001; Laurila & Seppa, 1998; Vieites et al., 2004). In this study, *K. eiffingeri* may oviposit in egg- or tadpole-occupied stumps, both resulting in overlapping offspring. However, parentage data and field evidence suggest that oviposition in stumps occupied by either eggs or tadpoles should be seen as two different reproductive phenomena, most likely with different causal mechanisms. Oviposition in egg-occupied stumps is mostly initiated by attending males which probably attempt to compensate for the loss of reproductive opportunities while attending eggs. On the other hand, based on the results of the nest choice experiment in the field, the occurrence of oviposition in tadpole-occupied stumps cannot be fully explained by the habitat saturation hypothesis. We propose several hypotheses to explain the potential adaptive values of overlapping offspring. However, additional studies are necessary to fully understand the patterns revealed by this study.

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