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RESEARCH PAPER

The Adaptive Significance of Egg Attendance in a South-East Asian Tree Frog

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Abstract

The arboreal frog, *Chiromantis hansenae* (Family: Rhacophoridae), is one of only a handful of South-East Asian amphibian species reported with parental care. We present the first systematic observational and experimental study confirming offspring benefits as a result of this care, which has a number of unusual life-history characteristics. Eggs are unusually small, breeding takes place in large pools, and females attend the eggs. Field observations and an adult removal experiment demonstrated a critical contribution of egg attendance to offspring survivorship. Harsh environmental conditions for offspring appeared to be the prime mover of parental care in this species, with desiccation as the main source of mortality when attending adults are absent. Results confirm females to be the caregivers, making *C. hansenae* a rare case of maternal egg attendance in a non-directly developing anuran.

Introduction

Parental care is behavior of a parent that increases survivorship of its offspring (reviewed in Clutton-Brock 1991). Wilson (1975) categorized environmental and ecological characteristics that drive evolution of parental care into four ‘prime movers’: (1) high levels of predation, (2) limited or rare food supplies, (3) unusually harsh environmental conditions, and (4) a stable and structured habitat. Aside from the more abstract notion of a structured habitat, each prime mover has been identified as a factor in the evolution of parental care in various taxa. For example, predation pressure triggers increase territorial defense in cardinal fish (Fukumori et al. 2009), nest protection in assassin bugs (Gilbert et al. 2010), and antipredator behavior in skinks (Huang & Wang 2009); limited food supply leads to brood ball protection in dung beetles (Halffter et al. 1996) and antimicrobial secretions to cover brood cells in digger wasps (Herzner et al. 2011); and harsh environmental condition drives nest shading behavior in shorebirds (AlRashidi et al. 2011), thermoregulatory care in dwarf hamsters (Wynne-Edwards 1998), and ventilation of brood pouches in amphipods (Tarutis et al. 2005).

Amphibians have remarkable diversity in reproductive ecologies (Duellman & Trueb 1994) and exhibit a wide array of parental care behaviors (Crump 1996), providing an excellent model system for investigating the costs and benefits, life-history trade-offs, and evolutionary drivers of parental care. Of Wilson’s four prime movers, predation pressure has been shown to have a stronger impact on parental care in some terrestrial breeding species (Bickford 2004), while harshness of environment appears to be the most important prime mover for arboreal breeding species (McDiarmid 1978; Bickford 2004). In other cases, limited food supply for tadpoles has been associated with trophic feeding, in which adults purposefully deposit unfertilized eggs as food source to sustain tadpole survival (Kam et al. 1996; Jungfer & Weygoldt 1999; Poelman & Dicke 2007).

At present, parental care has been documented in 17 of 27 anuran families (McDiarmid 1978; Crump 1996; Lehtinen & Nussbaum 2003), while new examples in those families continue to be reported (e.g., Gunther 2006; Ubeda & Nunez 2006; Sheridan & Ocock 2008). Eight different modes of parental care are generally recognized in amphibians: egg attendance, egg transport, egg brooding, tadpole attendance, tadpole transport, tadpole brooding, tadpole feeding, and froglet transport. Egg attendance, whereby a parent remains with the egg mass at a fixed location, represents by far the most common mode of amphibian parental care (Wells 1981, 2007; Crump 1996). By investing in egg attendance, amphibians can increase offspring survivorship by reducing or preventing developmental abnormalities (Forester 1979; Simon 1983), predation (Forester 1979; Kluge 1981; Townsend et al. 1984; Juncá 1996; Bickford 2004; Vockenhuber et al. 2009), and desiccation (Weygoldt 1980; Forester 1984; Taigen et al. 1984; Bickford 2004; Delia et al. 2013). In addition, it has been suggested that adults may reduce fungal infection of embryos during attendance (Salthe & Mecham 1974; Simon 1983; Green 1999). In particular, parental care in amphibians has been associated with five life-history traits: direct development (McDiarmid 1978; Summers et al. 2007), small clutch size and large embryos (Nussbaum 1985, 1987; Summers et al. 2006, 2007), lotic breeding habitats (Nussbaum 1985), and breeding sites with small water bodies (Brown et al. 2010). *Chiromantis hansenae* (Taylor 1962; Fig. 1), however, presents an unusual case that deviates from aforementioned patterns of life history and parental care. For example, *C. hansenae* has a relatively large clutch size (n = 150–250 eggs) and small eggs (1–2 mm egg capsule diameter) compared with other anurans with parental care (see Summers et al. 2007). It is not a terrestrial or direct-developing species and instead lays arboreal clutches overhanging lentic pools. In addition to these peculiar life-history characteristics, a previous report of *C. hansenae* suggests that egg attendance could be performed by females (Sheridan & Ocock 2008), which, if proven, would be a rare case among anurans without direct development, where the majority of egg attendance is performed by males (Crump 1996; Wells 2007). If so, *C. hansenae* would provide grounds for comparisons on the ecological and evolutionary significance of maternal versus paternal care and the costs and benefits for each sex.

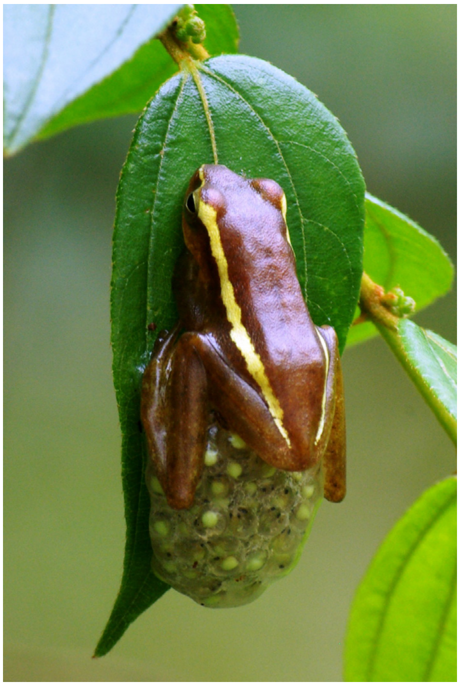

Fig. 1: *Chiromantis hansenae* female attending egg clutch.

The importance of gaining a better understanding of outlier species, such as *C. hansenae,* is exemplified by the fact that, thus far, the majority of research on amphibian parental care has occurred in the Neotropics, sub-Saharan Africa, and Melanesia, with a few cases in Europe and East Asia (Crump 1995, 1996; Wells 2007). In comparison, the situation in South-East Asia is poorly known. Of over 700 species (IUCN 2011), only a handful of species are reported to have some form of parental care (Inger & Voris 1988; Emerson 1996; Brown & Iskandar 2000; Sheridan & Ocock 2008). Moreover, these reports lack data quantifying any benefits of the supposed parental investment to offspring survival or costs to adult fitness. Assumed parental care may come from incidental observations of adult behaviors aimed at increasing mating opportunities rather than offspring survivorship (e.g., Woodruff 1977), making the gap between observations and analyses of costs and benefits one of the critical weaknesses in the study of parental care in amphibians (Crump 1996). These few South-East Asian species with parental care represent an opportunity to test theories developed from observations in other regions for ubiquity, as well as provide alternative and additional information that can be incorporated into future analyses to enhance our understanding of the evolution of such behaviors in general.

Herein, we examine the function and importance of egg attendance in *C. hansenae* as it relates to offspring survivorship via observation of (1) egg attendance behavior and embryonic mortality in undisturbed clutches, (2) embryonic mortality in paired adult removal experiments, and (3) sex of the caregiver. Through field observations and experimental manipulations of egg attendance, we determine whether parental care provides any direct benefits to offspring, consider the causes of offspring mortality in the absence of such behavior, and suggest factors that may be driving the evolution of parental care.

Methods

This study was conducted from July to October of 2011 and 2012 at the Sakaerat Environmental Research Station (SERS) in Thailand (14 30′ N, 101 55′ E, elevation of 250–762 m, mean annual rainfall of 980 mm). All observations were made in 2011 except for laboratory observations of amplectic pairs. *Chiromantis hansenae* is a nocturnal species and can be found breeding in temporary ponds mid-late rainy season. Eggs are deposited in a hemispherical gelatinous mass attached to vegetation or other substrates overhanging water. Embryonic period is 3–5 d, ending when tadpoles hatch and fall into the pond below.

Natural Egg Attendance Observations

Initial observation of new egg clutches was made between 03:00 and 06:00 h to ensure that clutches were located immediately after they were laid, the majority of which occurs between 21:00 and 03:00 h. Subsequent observations of clutches were conducted 4 times a day, at 6-h intervals, until all embryos had hatched, died, or disappeared.

Microhabitat characteristics recorded include distance between clutch and surface of pond when laid (hereafter ‘clutch height’) and type of material clutches were attached to (hereafter ‘breeding substrate’). Offspring survivorship was assessed by the presence or absence of embryonic mortality, which was used instead of counting the number of dead embryos to minimize human disturbance of attending adults and embryos. Causes of mortality were determined by examining the remainder of the clutch. Causes included predation, submergence, desiccation, and lack of development, where embryos were either unfertilized or failed to develop. Parental investment was quantified by percent of time attending adult was present during all observations for a given clutch. To control for abiotic factors, temperature and relative humidity were recorded using Onset HOBO® Pro v2 data loggers. In addition, daily rainfall was obtained from SERS weather stations records.

Adult Removal Experiments

Adult removal experiments were conducted to verify the benefits of parental care to offspring survivorship and to identify causes of offspring mortality. Experimental clutches, with attending adults removed, were paired with control clutches to minimize possible variations in embryonic development resulting from microhabitat and weather differences; pairs were made based on location (<100 m apart in oviposition site and <30 cm apart in clutch height) and time (<24 h apart in oviposition initiation). All clutches were attached to emergent vegetation to control for effects of breeding substrate. Data on microhabitat, offspring survivorship, and climate conditions followed those of natural observations, and all clutches were checked at 6-h intervals until clutches had hatched or died. Removed adults were kept in aquariums (25 × 40 × 25 height cm) in open air shed <2 km from original location following that of Institutional Animal Care and Use Committee (IACUC)-approved protocol (B11/12) and returned to original location immediately after clutch observations had concluded.

Sex of Caregiver

Sex of caregiving adult was determined by observing males and gravid females collected from the field and paired in open air laboratory settings following IACUC-approved protocol (B11/12). Females in amplectic pairs were identified by the presence of eggs seen through the abdominal membrane. Snout-vent length (SVL) of adults was measured to establish a baseline for male and female size. Once embryos hatched and observations of parental care concluded, tadpoles and adults were returned to their original locations.

To confirm sex of attending adults in natural field settings, SVL was measured for all adults removed in adult removal experiments. In addition, SVL of attending adults on clutches not included in natural observations was measured to confirm their sex. Adults attending clutches in natural observations were not measured to minimize possible disturbance that could affect parental care behavior or offspring survivorship.

Statistical Analyses

All statistical analyses were performed in R (R Development Core Team 2011). All means are presented with ±1 SE.

Offspring mortality in natural observations was analyzed with generalized linear models (GLM) with underlying Bernoulli distribution and logit link function. The Bernoulli distribution is a particular case of the binomial distribution applied to presence–absence data, in this case, the presence or absence of mortality in a given clutch. In a Bernoulli GLM, overdispersion cannot occur (McCullagh & Nelder 1989; Zuur et al.

2009). The effects of climate (temperature, relative humidity, and rainfall on day of oviposition), microhabitat (clutch height and breeding substrate), and parental investment (percent frequency of attending adult present) were tested in a single model. Offspring mortality in adult removal experiments was analyzed using 2 × 2 Fisher's exact test, used for small sample sizes, with clutch treatment (experimental or control) and clutch fate (hatched or died). Similarities in clutch height, temperature, and relative humidity between control and experimental group were tested using multivariate analysis of variance (MANOVA). Difference in adult SVL of males and females in amplectic pairs was tested using Welch's two-sample *t*-test.

Results

Field observations indicate that males call from vegetation or other substrate overhanging water to attract females, who then carry males on their back to an oviposition site. After all eggs are laid and presumably fertilized, males will leave while females continue to secrete mucus to form a thicker layer of gel covering the exposed surface of the entire clutch. Attendance behavior consisted of females primarily in close contact with clutches, covering part or all of exposed clutch surface with the posterior part of their body and hindlimbs. Occasionally, females would secrete liquid from their cloacae and extend their hindlimbs posteriorly to glaze the substance over the clutch. In addition, females were observed in a few instances to be resting on the side of the leaf or grass blade opposite to the egg mass. Adult attendance was observed at all stages of embryonic development. Although *C. hansenae* adults are nocturnal, females can remain with clutches during the day and maintain their position while resting. Attending females have, however, been observed to be absent from clutches during one observation and present for the next, indicating possible activities besides egg attendance during this period.

Increased Offspring Survival with Increased Care

A total of 126 clutches were observed, with up to 22 observations per clutch and amounting to 1448 observations in total. Of the observed clutches, 62.7% experienced mortality during the embryonic period, with predation being the primary cause of mortality (30.2%). Clutch predators included katydids, ants, and occasionally spiders. Other cases of mortality were associated with submergence (16.7%), lack of development (7.1%), desiccation (5.6%), and unknown causes (3.2%). On average, adults were present during 63.4 ± 2.4% of observations. GLM with all factors showed that parental attendance was the only significant factor in determining offspring survivorship (GLM, $X_1^2 = 29.43$, $p < 0.0001$, Fig. 2). Furthermore, all clutches where adults were never observed (0% attendance) died before embryos were able to hatch (n = 10). Clutch height was 40.8 ± 3.9 cm, and no significant correlation with clutch mortality was found (GLM, $X_1^2 = 1.57$, $p = 0.21$). Breeding substrate was not correlated with clutch mortality (GLM, $X_3^2 = 0.87$, $p = 0.83$), possibly due to the predominance of clutches on emergent vegetation (n = 106). In comparison, fewer clutches were observed on leaves or branches on trees (n = 16) and only occasionally seen on sides of rocks (n = 3) or a log (n = 1) overhanging water. Finally, temperature, relative humidity, and rainfall did not have an effect on clutch mortality (GLM, $X_1^2 = 1.60$, $p = 0.21$, $X_1^2 = 0.06$, $p = 0.81$, and $X_1^2 = 0.58$, $p = 0.45$, respectively).

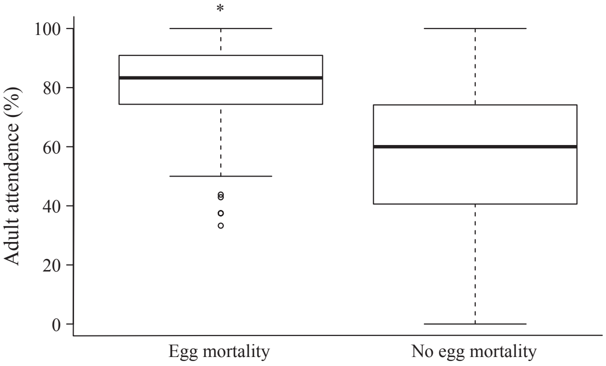

Fig. 2: Percent of time in natural field observations where attending adult was present for a given clutch. 'Egg moratilty' denotes clutches that exhibited offspring mortality, while 'No egg mortality' denotes clutches that hatched successfully without offspring mortality.

Obligatory Parental Care

Twelve paired adult removal experiments were conducted. Survival was significantly higher in control clutches with attending adults compared with experimental clutches with adults removed, with 9 of 12 control clutches hatching successfully, while all 12 experimental clutches ended in mortality (Fisher's exact test, n = 24, $p < 0.001$). Pairing of clutches ensured that treatment groups did not differ in clutch height, temperature, relative humidity (MANOVA, $F_{3,20} = 0.92$, $p = 0.45$, Wilk's lambda with type II sum of squares). Causes of clutch mortality in experimental

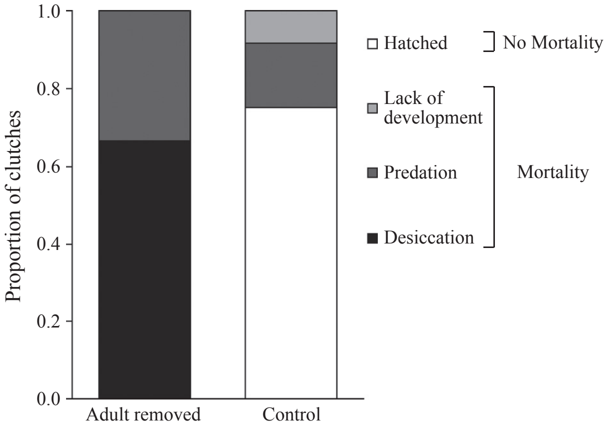

Fig. 3: Fate of *Chiromantis hansenae* egg clutches in adult removal experiment. All clutches in experimental group, with attending adults removed, died of mortality, while only 25% of control clutches died of mortality. Causes of mortality included desiccation (n = 8) and predation (n = 4) in clutches without adults and predation (n = 2) and lack of development (n = 1) in control clutches.

clutches were desiccation (n = 8) and predation (n = 4). In control clutches, mortality resulted from predation (n = 2) or instances where embryos were undeveloped (n = 1); however, no control clutch showed any sign of desiccation (Fig. 3). All embryos in experimental clutches died within 48 h after removal of the attending adult.

Maternal Egg Attendance

Laboratory observations of amplectic pairs of *C. hansenae* (n = 54) indicated that females remained with clutches, whereas males left once eggs were laid. Measurements of SVL showed that males were significantly smaller than females (male = 21.1 ± 0.1 mm, female = 24.5 ± 0.1 mm, Welch's two-sample *t*-test, $t_{101} = 23.24$, $p < 0.0001$) and did not exceed 23 mm. As mature female SVL was larger than 23 mm, this measurement was used to identify individuals performing egg attendance in natural settings. All attending individuals from adult removal experimental clutches were confirmed to be female using SVL (n = 12, SVL = 24.9 ± 0.2 mm). Similarly, all additional adults attending clutches in the field examined were female (n = 20, SVL = 24.9 ± 0.2 mm).

Discussion

Our results highlight harsh environment for offspring, one of Wilson's prime movers, as the main driver of parental care in an arboreal frog. Both natural observations and adult removal experiments in the field confirmed the benefits of egg attendance in *C. hansenae,* the former showing parental care as the main factor in determining offspring survivorship (Fig. 2) and the latter indicating that egg attendance is obligatory in aiding offspring survival and its primary function is to prevent egg desiccation (Fig. 3). In comparison, high levels of predation in natural observations indicated that although offspring predation could be correlated with certain aspects of parental care, it is unlikely to be the primary driver of parental care in *C. hansenae*. Finally, observations confirmed previous suggestions of females as caregivers in *C. hansenae* (Sheridan & Ocock 2008), presenting a rare case of female egg attendance in an arboreal breeding frog without direct development (see Wells 2007).

Moving toward more terrestrial breeding along an evolutionary trajectory of amphibians has led to diverse reproductive modes whereby eggs are laid outside of aquatic environments (Duellman & Trueb 1994; Wake 2003; Wells 2007). However, due to high membrane permeability of amphibian eggs, one of the challenges of eggs laid in non-aquatic habitats is its increased susceptibility to desiccation (Touchon & Warkentin 2008). Consequently, adaptations associated with terrestrial reproduction, including larger egg size, occurrences in wetter–warmer regions, and parental care (Gomez-Mestre et al. 2012), may have evolved to reduce egg desiccation. Other adaptations, such as laying foam nests that can retain moisture and using leaves to cover clutches and to reduce surface area exposed, although not yet tested, may serve the same purpose. For some foam-nesting *Chiromantis*, the outer layer of foam can harden when environmental conditions are dry, while the inside remains moist, allowing embryos to develop and hatch (Wager 1965; Seymour & Loveridge 1994). As opposed to *C. hansenae*, congeneric species in the region (such as *C. nongkhorensis* and *C. doriae*) do not exhibit parental care and lay foam nests instead of gelatinous clutches. Unfortunately, as with most South-East Asian species, phylogenetic data are not available for *C. hansenae* and most congeneric species. However, Rhacophoridae phylogeny by Li et al. (2009) suggested species in the genus *Chiromantis* that lay gelatinous clutch are a sister clade to foam-nesting species. Contrary to foam nests, the lack of physiological or morphological adaptations to water loss in *C. hansenae* clutches may be correlated with behavioral changes in adults (i.e., the appearance of egg attendance). Of course, there can be a number of factors contributing to the division between parental care for gelatinous clutches of *C. hansenae* and no parental care for foam nests of

C. nongkhorensis and *C. doriae*; however, preventing water loss seems to be one logical explanation. This is suggested by the results in adult removal experiments showing high levels of egg desiccation in the absence of attending adults, whereas control clutches were buffered from this source of mortality (Fig. 3), supporting Wilson's (1975) hypothesis that harsh environments can be a driver of parental care.

Prevention of water loss has been cited as a major function of egg attendance in amphibians, and it has been suggested that adults may (1) reduce surface exposure and thus evaporation in embryos (Forester 1984), (2) move water osmotically from their ventral integument to embryos (Taigen et al. 1984; Bickford 2004), (3) bring water to embryos by repeatedly soaking themselves in water and placing their bodies over clutches (Ueda 1986; Cheng & Kam 2010), and (4) actively release internal fluids over embryos to increase hydration (Weygoldt 1980, 1987; Bourne 1998). Observations of *C. hansenae* indicate that it may be using a combination of these methods, including reducing surface exposure by physically covering the clutch and possibly secreting liquids to maintain or increase hydration of embryos. As suggested in Wilson's harsh environmental conditions for offspring, the presence of parental care in *C. hansenae* mainly functions to protect gelatinous clutches from desiccation. Alternatively, closely related species that lay foam nests that provide more nourishing microenvironments for embryos lack this behavioral adaptation.

One important aspect that distinguishes egg attendance in *C. hansenae* from previous findings is the sex of adults providing care. Male parental care may be the typical primitive form of parental care in other anurans (Brown et al. 2010), and reports of male egg attendance far outweigh female egg attendance in species without direct development (see Wells 2007). Unlike the majority of known examples, however, females of *C. hansenae* are the ones providing egg attendance. Although certain costs, such as increased adult exposure to predators (McDiarmid 1978; e.g., Reguera & Gomendio 1999; Burris 2011), can be present for both sexes, others may be more sex specific. For species that exhibit paternal egg attendance, males can care for multiple broods (Taigen et al. 1984; Wootton 1984; Juncá 1996; Burrowes 2000; Manica & Johnstone 2004; Delia et al. 2013), seek additional mating opportunities (Juncá 1996; Cheng & Kam 2010; Delia et al. 2013), or even increase their attractiveness to potential mates by exhibiting brood care (Knapp & Sargent 1989; Forsgren et al. 1996; Forsgren 1997; Reynolds & Jones 1999; Reynolds et al. 2002; Nazareth & Machado 2010) (but see Gomendio et al. 2008). As opposed to paternal egg attendance, maternal egg attendance may directly reduce future reproductive output of females via reduction in mating opportunities and resource acquisition. Because there are no previous studies on female egg attendance similar to *C. hansenae*, we can only hypothesize on costs to females by comparisons with other species. Females in *C. hansenae* only care for one clutch at a time and therefore cannot increase reproductive output via additional mating during attendance. As attendance period is relatively short in *C. hansenae*, however, reduced mating opportunities may be less of a cost for females compared with limits on their ability to produce gametes. Whereas reproductive success in males is limited by mating opportunities, female reproductive success is dependent on gamete production (Trivers 1972), which is directly related to body size (Gross & Sargent 1985) and would presumably be negatively affected by investment in parental care. In anurans with direct development and prolonged periods or high frequency of egg attendance, adults can lose a significant fraction of their body mass and abdominal fat bodies while attending their clutches (e.g., *Cophixalus parkeri* 85–100 d, *Eleutherodactylus coqui* 20 d) (Simon 1983; Townsend 1986). Possible decrease in body mass in *C. hansenae* females, although potentially small considering its short embryonic period (5 d), could still represent a substantial cost, because females do not forage while in attendance (*pers. obs.*). Of course, costs of egg attendance can vary greatly for either parent, both between and within species, depending on time and energy invested. To further complicate matters, even in cases where adults can care for multiple clutches, it is not necessarily correlated with increase in adult fitness (Cheng et al. 2013), on top of which individuals could be providing care to unrelated embryos (Chen et al. 2011). It is clear, however, in the case of *C. hansenae* that benefits of egg attendance exceed costs, as clutches are unable to develop without attending females. As this is the first systematic observation and experimental confirmation of parental care in *C. hansenae*, much of the costs associated with this behavior and its implications for the evolution of maternal versus paternal care require further investigation. For instance, antipredator behavior against katydids has been observed in attending females in natural settings, which could signify a substantial cost for females as katydids present predation risks to both egg clutches and adults. Nonetheless, our results quantify and demonstrate the importance of female egg attendance in *C. hansenae* and provide new grounds for comparisons, as it denotes a deviation in reproductive strategies from closely related species within the region and presents information on a species from a region that is under-represented in our current knowledge of amphibian parental care.

In conclusion, this study confirms Wilson's theory of harsh offspring environment as a primary driving force for parental care in an arboreal frog. We highlight the critical role of parental care in *C. hansenae* in preventing egg desiccation and possible mechanisms of buffering environmental pressures for offspring. We present an experimentally confirmed case of female egg attendance in a non-direct-developing anuran, which has implications for residual reproductive output and sexual selection. Finally, we point out potential areas of research that may lead to novel understandings of the evolution of parental care and decisions involved in adult care behavior.

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