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Spawning behaviour of *Engystomops pustulatus* (Anura: Leptodactylidae)

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ABSTRACT

Foam nests have evolved independently in several amphibian groups as an adaptive response to prevent predation and desiccation in dry environments. Nests are normally laid on ponds, or in underground galleries, humid forest leaf litter or terrestrial bromeliads. They are built when males or females beat a foam precursor associated with the egg masses extruded by the female. The spawning process requires the synchronic actions of the mating pair to obtain a hemispheric nest that protects the offspring. Herein, we describe the spawning behaviour of *Engystomops pustulatus* based on videos from 13 nesting couples from the lowlands of western Ecuador. Three variables were measured as indicators of male effort: duration of mixing events, duration of resting periods, and number of kicks per mixing event. We consider that not only male physical effort but also female behaviour influences nest structure. We suggest that nest building requires prolonged and intense physical activity by the male as well as the female's steady position during spawning and female's oviposition site selection. Nest building has two phases. In the first phase, the duration of resting periods, the duration of mixing events, and the number of kicks increase and are highly variable. During the second phase the three variables stabilise until the end. The volume of the nest increased mainly during the second phase. In four nesting events we observed kicking movements by the female. To our knowledge, this is the first time that female kicking has been observed in leptodactylid frogs. The function of this behaviour is unknown but our observations suggest that it may be triggered by insufficient male effort. Traditionally, female mate choice in *Engystomops* has been explained under models of indirect benefits exclusively. We argue that the prolonged male activity during nesting could influence female fitness directly. This will allow the operation of sexual selection via direct benefits.

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Engystomops; foam nest; kicking; mixing events; spawning

Introduction

During early developmental stages, amphibians are highly vulnerable to predation and desiccation. A great diversity of reproductive modes have evolved across the group to cope with these challenges (Duellman and Trueb 1986; Schalk and Saenz 2016). For

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Supplemental data for this article can be accessed here.

instance, several amphibian families contain species that oviposit eggs in foam nests: South American Leptodactylidae, Afro-Asian Rhacophoridae, Australian Limnodynastidae and African Hyperoliidae (Tyler and Davies 1979; Duellman and Trueb 1986; Haddad et al. 1990; Haddad and Prado 2005; Faivovich et al. 2012). The foam nest has evolved to protect the eggs from aquatic predators and from desiccation when they are exposed to drought; in addition, it provides nourishment for the hatchlings until they develop into tadpoles (Ryan 1985; Haddad and Prado 2005; Wells 2007; Fleming et al. 2009). These nests are built on water surfaces in ponds, in underground galleries, on humid forest floors or in terrestrial bromeliads (Heyer and Rand 1977; Duellman and Trueb 1986; Zina 2006). The foam is formed when either the female or the male beats the foam precursor with her forelegs or his hind legs, respectively. While 'external fertilisation' takes place through amplexus, the female discharges through the cloaca the precursor jelly. This precursor is a cocktail of six proteins named ranaspumins, which are harmless to eggs and confer resilience to the nest (Fleming et al. 2009; Faivovich et al. 2012).

Within Leptodactylidae, all the species of *Engystomops* lay foam nests in temporary ponds. They are associated with disturbed areas in primary and secondary forests from Central and South America (Heyer and Rand 1977; Ryan 1985; Funk et al. 2007, 2011; Dalgetty and Kennedy 2010; Ron et al. 2014). The spawning behaviour of *Engystomops* has been widely evaluated; however, only Dalgetty and Kennedy (2010) provide for the first time a detailed description of the nest-building process for túngara frogs (*E. pustulosus*). In these species, males form the foam with periodic movements of their hind legs, mixing the jelly extruded by the female during amplexus. Kicking bursts are interrupted by intervals of leg immobility (Heyer 1969; Crump 1974; Ryan 1985). Throughout this article, we refer to the kicking burst as a 'mixing event', and to the immobile interval as a 'resting period'. The combination of the two events represents one kicking cycle. Dalgetty and Kennedy (2010) suggest a three-phase construction process in *E. pustulosus*. The first phase is characterised by an increase in the duration of mixing events and an irregular duration of resting periods without egg deposition. In the second phase there is constant duration of mixing events and resting periods. The third phase shows an exponential increase in the duration of the resting period. This process generates a nest with a core chamber, where the eggs are concentrated, and a distinct foam cortex without eggs (Dalgetty and Kennedy 2010).

Engystomops pustulatus is distributed in the dry and tropical forest of western Ecuador and Peru (Ron et al. 2014). They breed during the rainy season; however, despite the abundant water resource due to constant rain, tadpoles from several populations are at high risk of desiccation. They occupy mainly disturbed areas where temporary ponds dry out rapidly during daytime (Ron et al. 2014). Ron et al. (2014) describe partially the spawning behaviour of *E. pustulatus* and suggest that, similar to other *Engystomops* species, nest building represents a high energy investment for males and that their physical effort decreases as the nest building process goes on. Herein, we analyse male and female behaviour during spawning of *E. pustulatus*, in order to determine the actual physical contribution of each parent for the resulting nest structure.

Materials and methods

Pairs of *Engystomops pustulatus* in amplexus were collected from temporary ponds in gravel streets or next to agricultural fields in Patricia Pilar (Province Los Ríos, Ecuador; 0.53717°S, 79.37071°W; 200 m above sea level). Collections took place between February and April 2008. Observations of nesting behaviour were made a few hours after capture. We placed amplectant pairs in plastics containers of 500 mL (10.6 × 6.1 cm) with 1 cm (75 mL) of fresh water; pairs were kept in darkness and under local environmental conditions (23.2–35°C air temperature and 24–27°C water temperature). Some frogs were maintained in captivity for up to 6 months at Balsa de los Sapos, Museo QCAZ. During that period, they nested frequently and those nesting events were also used for our spawning descriptions. Frogs kept in the laboratory were maintained in large terraria with at least 1 cm of water (environmental conditions 24–26°C air and 23–24°C water). We maintained frogs on a normal 12:12 h light/dark cycle. Terraria were kept behind a plastic barrier that isolated frogs from the observer and external lights that could have disturbed the animals. The nest-building process of 13 pairs (10 in the field and three in captivity) was recorded with Sony TRV70 and Sony TRV18 video cameras. Videos were digitalised with iMovie version 6.03 and Final Cut Pro version 5.0. Behavioural sequences were analysed using the software JWatcher, version 1.0 (Blumstein Inc. 2000–2012). We evaluate three variables as indicators of male effort: mixing events, resting periods, and number of kicks per mixing event. In order to compare the spawning process with the phases described for *Engystomops pustulosus* by Dalgetty and Kennedy (2010), nest building was divided into phases with split-points corresponding to sudden changes in the duration of mixing and resting events. A Wilcoxon signed rank test was used to compare the periods of nest building for the three variables that measure male effort.

We also classified nest spawning in two groups based on females' intervention: Nests built by male kicking with no female movement (Figure1a) and nests built by both parents, male and female, kicking (Figure 1b). A Mann-Whitney U test was used to evaluate whether the number of kicks per cycle and the duration of mixing and resting events vary due to female intervention. Analyses were carried out using IBM SPSS Statistics for Macintosh (version 20.0).

Results

The total duration of the spawning process varied across couples, ranging from 37.1 to 223.3 minutes (Table 1). Nests built in the field and in the laboratory were analysed as one group because no significant differences in the sequence of nest building were found: mixing events ($U = 49$, $p = 0.53$), resting period ($U = 72$, $p = 0.49$) and number of kicks ($U = 35$, $p = 0.14$; $N = 13$).

Once amplexus takes place, the female walks around the pond until choosing a spawning place, always covered with water. Once she stops walking, the female kicks the substrate repeatedly to make a shallow hole, and the male starts kicking. We observed a reduction in the speed of male kicks at the end of every mixing event (last 1–3 kicks) and changes in direction of leg movement from primarily longitudinal (front to back) to primarily lateral (side to side). During side-to-side kicking, the hind legs are almost completely extended and remove part of the foam that lies immediately behind

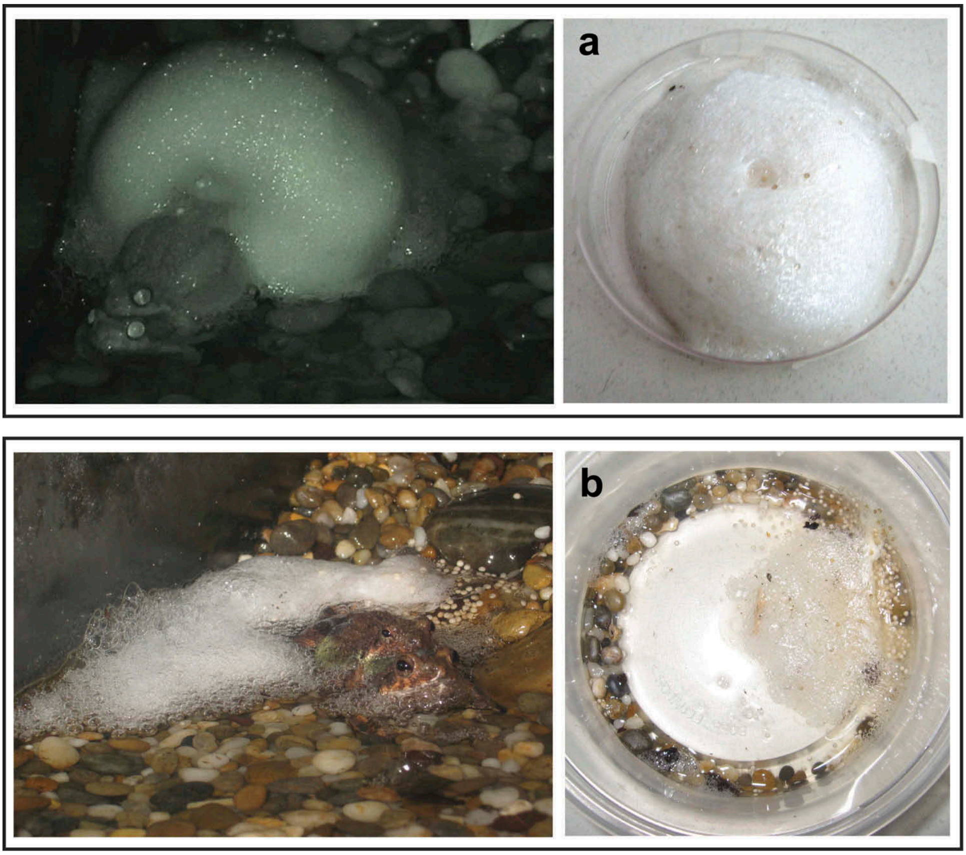

Figure 1. Foam nest type according to intervention of females: (a) nest built by males kicking with no female intervention, (b) nest built including males' and females' kicking movement. Structure of the nest during spawning process is presented on the left, and the final nest shape after spawning on the right.

the vent (Figures 1a and 2). We could not identify an inflection point to delimit a third phase as in Dalgetty and Kennedy (2010); therefore, only two phases were defined (Figure 2). The first phase begins when the male initiates kicking, and is characterised by high variation in the three measured variables (mixing event duration range 0.56–28 seconds, resting period duration range 1.26–254 seconds, number of kicks range 2–38). The second phase starts when the three variables reach a plateau with lower variation and more constant values (Figure 2; mixing event duration range 1–4.87 seconds, resting period duration range 1.14–30.17 seconds, number of kicks range 1–31). We found significant differences between the first and second phases: Wilcoxon signed rank test: mixing event duration (W = 91, p = 0.001), resting period duration (W = 10, p = 0.013) and number of kicks (W = 91, p = 0.001 N = 13). Only 19% (range 4–38) of mixing events took place during the first phase (Figure 2). Videos of the spawning process are provided as supplementary materials.

Interestingly, we observed females from four couples (8 F, 10 F, 12 L, 13 L in Table 1) kicking slowly and laterally with their hind legs (2 to 3 times per cycle) in a similar manner to males. These movements pushed the male's hind legs and reduced males'

Table 1. Male physical effort evaluated during spawning. F represents a nest in the field and L a nest in the laboratory. Total time of processes is reported in minutes, and time per cycle is reported in seconds.

	Total nest measurements					Measurements per event (seconds)			
Nest number	Resting time (min)	Kicking time (min)	Number of kicks	Spawning duration (min)	Number of kicking cycles	Resting time (s)	Kicking time (s)	Number of kicks	Number of cycles per minute
1F	48.76	20.49	146	69.26	187	11.94	5.02	36	3
2F	54.05	24.49	201	78.53	370	8.76	3.97	33	5
3F	52.86	19.56	143	72.42	322	9.87	3.64	27	4
4F	63.85	28.91	186	92.76	409	9.39	4.24	27	4
5F	38.71	11.69	104	50.39	196	11.85	3.57	32	4
6F	117.02	24.72	217	141.74	421	16.51	3.48	31	3
7F	66.95	26.39	180	93.35	375	10.66	4.2	29	4
8F	92.84	21.78	157	114.63	331	16.43	3.86	28	3
9F	41.10	17.99	134	59.09	242	10.23	4.48	33	4
10F	60.94	9.00	80	69.94	167	21.64	3.18	29	3
11L	29.66	7.43	59	37.09	126	14.24	3.54	28	2
12L	194.12	29.18	236	223.30	548	21.14	3.17	25	4
13L	77.34	25.40	164	102.74	357	13	4.27	28	3
Mean and SD (min)	72.17 ± 43.49	20.54 ± 7.24	154 ± 52	92.71 ± 48.08	312 ± 121	13.51 ± 4.28	3.89 ± 0.54	30 ± 3	4 ± 1

SD: standard deviation.

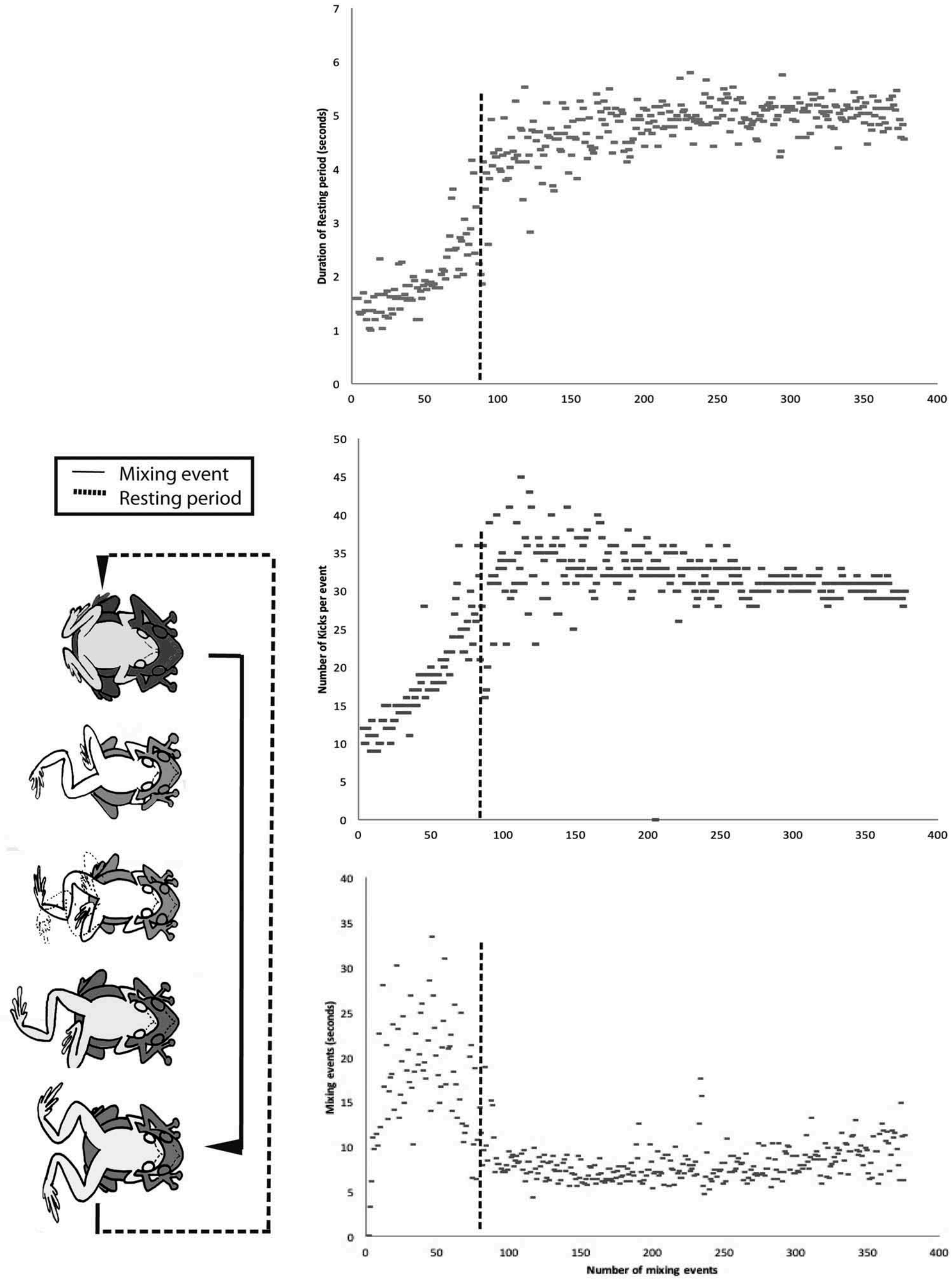

Figure 2. Full spawning sequence of *Engystomops pustulatus* (Pair 7F, Table 1). Right charts indicate duration and number of the mixing event and the resting periods and the number of kicks. Left panel diagram exemplifies one kicking cycle; arrows point out the direction of the sequence and dashed line shows the transition point-mark between the first and second phases (Mixing event no. 83). Drawing by Liliana Jaramillo.

kicking effort. There were significant differences in the duration of resting periods (U = 3, $p = 0.025$) between nests built with female intervention (longer duration) and nests built without female movements; no significant differences were observed in the number of kicks per cycle (U = 14, $p = 0.32$), nor in kicking duration (U = 11, $p = 0.59$). Female leg movements resulted in nests with non-spherical structure (Figure 1b). In two of these cases (couples 8 C and 10 L), females moved to a different position within the nest and males stopped kicking. When females stopped, after having changed position several times and moved for a long period (between 10 to 25 minutes), males started kicking again and the number of kicks per mixing event increased gradually until reaching a stable rate and the final shape of the nest was spherical as well (Figure 1a).

Discussion

Three factors seem to determine the nest structure: kicking effort by the male, female position during spawning and female selection of a nesting site. We show that nest building requires prolonged and intense physical activity by the male. Both the effort throughout the duration of spawning and the change in kicking direction and speed towards the end of each mixing event appear to contribute to the hemispheric shape of the nest. The transition between the first and second phase was evidenced by stabilisation in mixing event duration, resting period duration, and the number of kicks per mixing event. Unlike the *E. pustulosus* study, no significant change in these variables was detected during the last third of nest building. In our observations during the first phase, male effort was highly variable. Kicking time and number of kicks increased while resting time decreased. Similar to the *E. pustulosus* study (Dalgetty and Kennedy 2010), the second phase is the most stable and accounts for most of the male's physical effort; nevertheless, the third phase characterised by the increase in duration of mixing events was not observed in *E. pustulatus*. The lateral kicks push the foam backwards and generate space between the male's hind legs and female's cloaca between cycles, moving the foam further. The building process had two phases and was less complex than the three-phased process observed in *E. pustulosus* (Dalgetty and Kennedy 2010). Our observations of male kicking behaviour are consistent with the behaviour reported by Ron et al. (2014).

Passive behaviour by females also seems to be important for nest building. We believe that female movements during nesting may prevent foam formation. Our observations indicate that when the female kicks or walks, the spawning process is lengthened or a non-hemispheric final nest structure is obtained. Previous studies on *E. pustulosus* and *Physalaemus centralis* indicate that, similar to our observation of motionless females spawning, females remain still during the process in order to produce a well-structured nest (Heyer and Rand 1977; Brasileiro and Martins 2006). Although Heyer and Rand (1977) and Ron et al. (2014) reported female abdominal squeezing before every mixing event in *E. pustulosus* and *E. pustulatus*, respectively, no kicking movements have been previously described. Our study is the first to report female kicking movements during spawning. The function of this behaviour is unknown; presumably, females mix the foam precursor matrix if there is insufficient male effort, yet further studies are required to test this hypothesis.

We suggest also that females' selection of oviposition site contributes to the formation of a hemispheric nest. Sexton and Ortleb (1966) postulated that males select an oviposition site before they start calling, based on the need for a solid substrate for the pair to anchor during spawning. On the other hand, Dillon et al. (2000) suggested that it is the female who selects the oviposition site, once the amplexus has taken place. We observed amplectant females moving and changing position before and, in a few cases, during the spawning process. During this period, the male is fully supported on the female's body, creating a need for anchorage for herself to maintain the same posture until the end of the spawning. Therefore, we support the notion of female selection of oviposition site.

We are sure that female movements were not produced as a response to the presence of intruders or the observer. Our study is one of the few to make detailed descriptions of nesting behaviour without disturbing the animals with artificial light. The artificial light used for observations of nocturnal anurans could affect or modify their behaviour given that they may be active at lower light levels compared to human perception (Buchanan 1993). Our observations were made with infrared light, under dark conditions and with a barrier isolating the observer. Therefore, we avoided interfering in the spawning process.

Studies of sexual selection in these frogs have assumed that female mate choice is driven exclusively by indirect benefits (Ryan 1985). Undoubtedly, males' energetic contribution during nesting has a direct influence on offspring survival, and could be highlighted as a direct benefit. Further studies evaluating the relationship between female choice, male investment, and offspring survival are needed to evaluate this hypothesis.

In summary, *E. pustulatus* couples spawn similarly to their congeners; but, unlike *E. pustulosus*, *E. pustulatus* exhibits a simpler nesting behaviour consisting of two phases. Not only male physical effort but also female behaviour contributes to produce a hemispheric nest shape. We suggest that hemispheric nests will prevent desiccation, hence increasing offspring survival; however, further evaluation of the role of nest shape is required to clarify this statement. To our knowledge, this is the first time that female kicking has been observed in leptodactylid frogs spawning. The function of this behaviour remains unknown.

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Disclosure statement

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