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Reproduction and spawning behavior in the frog, *Engystomops pustulatus* (Shreve 1941)

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***Abstract.*—The study of reproductive strategies is central to understand the demography of populations and the energetic relationships of the species with their ecosystem. Documenting the reproductive natural history of the species is pressing in groups, like amphibians, that are threatened with extinction at a global scale. Herein, we describe the reproductive ecology and spawning behavior of the leptodactylid frog *Engystomops pustulatus*. In addition, we report observations that suggest the existence of an alternative mating strategy. Our results show that reproduction in *E. pustulatus* is characterized by high maternal investment (15% egg mass relative to body mass). We found evidence of size-assortative mating with a tendency of larger females to mate with larger males. Clutch size was correlated with female weight, female condition and male size. Larger females showed a tendency to lay larger foam nests and larger nests contained more eggs. At reproductive choruses, there was a male-biased operational sex ratio, indicative of high variance in male reproductive success. We observed an amplectant couple spawning while an additional male was embedded in the foam. We hypothesize that this behavior is evidence of an alternative mating strategy where a small non-amplectant male attempts to fertilize the eggs that are extruded by the amplectant female.**

***Resumen.*—El estudio de las estrategias reproductivas es fundamental para entender la demografía de las poblaciones y las relaciones energéticas de las especies con su ecosistema. Documentar la historia natural reproductiva de las especies es apremiante en grupos, como los anfibios, que están amenazados con extinción a nivel mundial. Aquí, describimos la ecología reproductiva y el comportamiento de anidación en la rana leptodactílida *Engystomops pustulatus*. Además, reportamos observaciones que sugieren la existencia de una estrategia reproductiva alterna. Nuestros resultados indican que la reproducción en *E. pustulatus* está caracterizada por una alta inversión energética de la hembra (15% de masa de huevos en relación a la masa corporal). Se evidencia que el apareamiento es selectivo con respecto al tamaño, con una tendencia de hembras grandes a aparearse con machos grandes. El tamaño de la puesta estuvo correlacionado con el peso de la hembra, la condición de hembra y el tamaño del macho. Las hembras más grandes mostraron una tendencia de poner nidos de espuma más grandes y los nidos más grandes tuvieron un mayor número de huevos. En coros reproductivos, hubo una tasa sexual operativa sesgada hacia los machos, lo que indica una alta varianza en el éxito reproductivo de los machos. Se observó una pareja en amplexus construyento un nido mientras un macho adicional estaba incrustado en el nido de espuma. Hipotetizamos que este comportamiento evidencia una estrategia de apareamiento alterna en la que un macho pequeño intenta fertilizar huevos puestos por una hembra en amplexus con otro macho.**

Key words. Alternative mating strategy, clutch size, clutch piracy, fertilization rates, nesting behavior, testis size

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Introduction

Understanding the natural history of reproduction is essential to characterize the ecological niche and the survival prospects of amphibians. Acquiring a better understanding of amphibian reproduction will assist conservation efforts in the vertebrate class with the higher number of species threatened with extinction (Chanson et al. 2008).

The deposition of eggs in foam nests characterizes the reproduction of most species of the Neotropical family Leptodactylidae, which has 201 species distributed from southern Texas to southern Chile (Frost 2014). In most species, males call in choruses that are visited by receptive females, which then actively choose a mate (Ryan 1985). Amplectant pairs build foam nests where hundreds of eggs are laid and fertilized (Crump 1974; Heyer 1969; Ryan 1985). The foam is formed when the male kicks the jelly surrounding the eggs while the female discharges them. The foam may protect the eggs from dehydration and/or predation (Duellman and Trueb 1994; Menin and Giaretta 2003) or from excessive heat (Gorzula 1977).

Foam nests may facilitate multiple paternity by retaining sperm (Kusano et al. 1991). There is a high proportion of foam-nesting species among known cases of multimale spawning in anurans, eight out of 15 (Byrne and Roberts 1999; Kaminsky 1997; Prado and Haddad 2003). Although several reproductive characteristics of Leptodactylidae should favor multiple male mating strategies, there are only two documented cases, *Leptodactylus chaquensis* and *L. podicipinus* (Prado and Haddad 2003). The paucity of records may be partly due to lack of studies. Although several leptodactylid species are abundant and live even in urban areas, little is known about its biology beyond brief accounts of its systematics and morphology. Such is the case of the widely distributed and abundant *Engystomops pustulatus* (Ron and Read 2012).

Herein, we describe the reproductive natural history of *Engystomops pustulatus* including fertilization rates, testis size, clutch size, and relative egg mass to explore factors that influence mate choice and reproductive output. We also describe its spawning behavior with observations suggest the existence of a secondary male mating strategy.

Materials and Methods

Study site and species

Engystomops pustulatus inhabits dry shrub, deciduous forest, and lowland moist forest below 300 m in western Ecuador. It can be relatively common during the rainy season, when they reproduce. They are explosive breeders that congregate around temporal pools. Males call from the water and amplectant pairs build foam nests to deposit their eggs (Ron and Read 2012). *Engystomops pustulatus* should not be confused with *E. pustulosus*, a Central American species that has been a model for studies of behavioral ecology (e.g., Ryan 2005). For clarity, hereafter, we refer to *E. pustulosus* exclusively as "Túngara frog."

Operational sex ratio (e.g., the number of males relative to the number of females in breeding aggregations) in *Engystomops pustulatus* was assessed in western Ecuador at three localities: Reserva Cerro Blanco, (W 80.0214°, S 2.0264°, Provincia del Guayas; 19 March 2003), Patricia Pilar (Provincia Los Ríos; 21 February 2002), and the town of La Maná (Provincia de Cotopaxi; 28 December 2003). Reproductive output, nest size and size assortment were evaluated in La Maná (W 79.265°, S 0.943°, elevation 160 m) between 28 December 2003 and 08 February 2004 and Patricia Pilar (W 79.3707°, S 0.5372°, elevation 200 m) between 23 January and 20 April 2008 during the rainy season. At La Maná and Patricia Pilar, the vegetation is Evergreen Lowland Moist Forest (as defined by Sierra et al. 1999). Most of the forest in the region has been converted to pastures and agricultural lands. Field observations took place after dusk, between 19:00 and 3:00 h. Breeding occurred in small temporary ponds on the streets of the town. Some sites were under dim artificial light (street poles).

Fertilization rates and nest size

We estimated fertilization rates from amplectant pairs collected from the field. The amplectant pairs were placed in individual circular plastic containers (10 cm diameter) with water depth of one cm. Most pairs made a nest after few hours. Three or four days later, we washed the foam with a solution of chlorine and water and counted the number of hatched and undeveloped eggs (as described by Ryan 1983). We used this proportion as a proxy for fertilization rates. This methodology does not allow discriminating between undeveloped eggs as result of egg unviability or failed fertilization. Therefore, our methodology may slightly underestimate fertilization rates.

To estimate nest size, we measured (with digital calipers, to the nearest 0.01 mm) the length of the longest axis, width at the widest point perpendicular to the longest axis, and height of all nests laid in the containers. We estimated nest volume with the formula of ½ ellipsoid:

$$V = \frac{\pi}{12} abc$$

where a, b, and c are the length, width, and height, respectively. The measurements were taken while the nests were <1 day old.

Adult size and egg mass

Sex was determined by the presence of nuptial pads, vocal sac folds, and/or by gonad inspection. Snout-vent length (SVL) was measured with Fowler digital calipers (nearest 0.01 mm). Body mass was measured in the field

(before and after oviposition in females) with a digital balance (nearest 0.1 g). Relative egg mass (maternal investment) was calculated as 1—the ratio (female mass after oviposition/female mass before oviposition).

After being kept in the plastic containers to allow spawning, females were euthanized by immersion in chloretone, fixed in 10% formalin, and preserved in 70% ethanol. Egg mass and body mass were measured after preservation in females that did not spawn. Each female was weighted on a digital balance (to the nearest 0.001 g), after removing excess ethanol. Then, the remaining egg masses (including immature eggs and jelly) were removed from the abdomen and weighted. Relative egg mass was calculated by dividing total egg mass by non-gravid female mass. Estimates of relative egg mass could be influenced by preservation in ethanol. Therefore, comparisons with relative egg mass in non-preserved nesting females should be interpreted with caution. All preserved specimens are deposited at the amphibian collection of the Zoology Museum of Pontificia Universidad Católica del Ecuador.

Reproductive behavior

Behavioral observations were carried out at male choruses in La Maná, Ecuador. Spawning behavior was described from of a single nesting event at La Maná. Spawning was recorded in the field under infrared light with a digital camcorder SONY TRV70. The complete video is available at AmphibiaWeb (http://amphibiaweb.org).

Statistical analyses

For normally distributed variables, we tested the significance of relationship between them using linear regression ANOVAs; for non-normal variables, we tested relationship with Spearman's rank correlations. Differences between groups were tested with *t*-tests (assuming non-equal variances). Statistical tests were implemented in software JMP v.5.1 (SAS Institute, 2003).

Results

Reproductive output, fertilization rates, and nest size

Among 77 nests, the mean number of eggs was 320 (SD = 142.6, range 0–747). The average percentage of unfertilized eggs was 1.89% (SD = 3.3, 0–19.1, n = 46); Fig. 1A); ~1/5 of the nests had a fertilization rate of 100%. Snout-vent length difference between both parents was not correlated with the number of unfertilized eggs (Spearman's Rho = 0.098, P = 0.524) or the proportion of unfertilized eggs (Rho = 0.145, P = 0.341).

Mean nest volume was 37.0 cm3 (SD = 14.4, range 2.8–85.6, n = 74). Nest volume is correlated to the number of eggs (larger nests have more eggs; Table 1, Fig. 2A) and female size (larger females lay larger

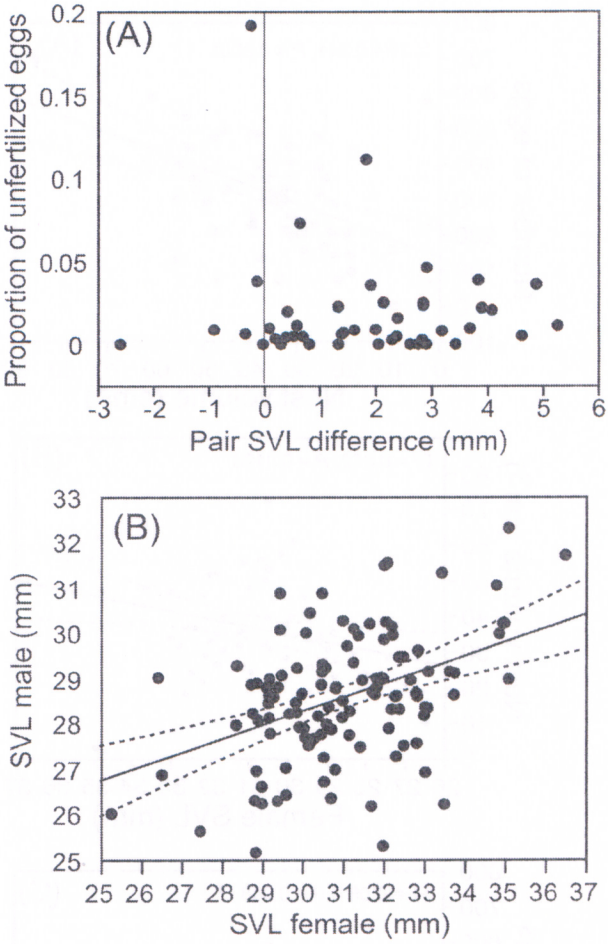


Fig. 1. Size and fecundity rates for amplectant pairs of *Engystomops pustulatus*. After collected in amplexus in the field, pairs were left in plastic containers where they could spawn. (A) Proportion of unfertilized eggs among pairs that successfully built a nest, (B) Female vs. male snout-vent length (SVL) with linear regression and 95% confidence intervals (dashed lines).

nests; Table 1, Fig. 2B). A multiple regression of number of eggs, female SVL, and male SVL explains 25% of the variation in nest volume (F = 7.51, df = 66, P < 0.001). However, only number of eggs is significant for the regression model (F = 18.18, P < 0.001).

Number of eggs was significantly correlated with male SVL (Fig. 2C) but not with female SVL. Number of eggs was correlated with female mass before and after oviposition and female condition (Table 1).

Non-spawning females had large masses of eggs in their abdomens (mean relative egg mass = 0.354, SD = 0.138, range 0.129–0.621, n = 13). Average maternal investment for spawning females was 15.2% of body weight (SD = 7.77, 1.8–39.4, n = 42).

Size assortment and spawning

We found size-assortative mating as male and female size of amplectant pairs was correlated (ANOVA's F = 24.1, P < 0.001, R^2 = 0.176; Fig. 1B). Overall, females were significantly larger than their mates (n = 115; mean female SVL = 31.0 mm, SD = 1.9, range 25.3–36.5; male

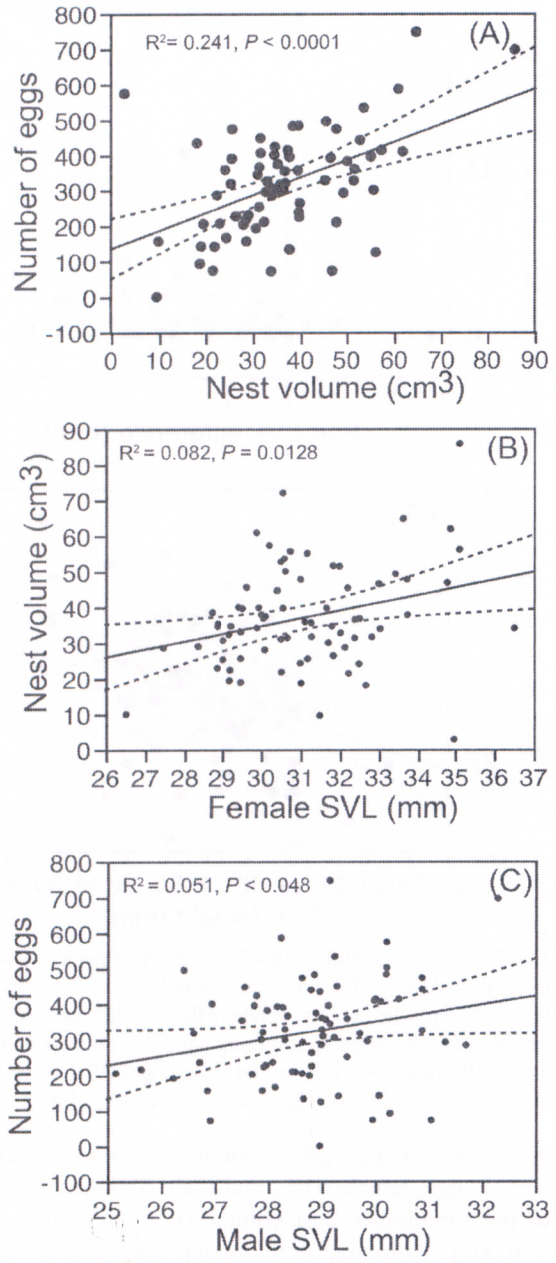


Fig. 2. Bivariate plots for (A) nest volume vs. number of eggs, (B) females size vs. nest volume, and (C) male size vs. number of eggs in *Engystomops pustulatus*. Linear regressions with 95% confidence intervals (dashed lines), determination coefficients (R^2), and ANOVA's *P* values are shown.

SVL = 28.5, SD = 1.3, 25.2–32.3; paired-*t* = 14.7, df = 104, $P < 0.001$). However, in 10 pairs (8.6%) the male was larger. Mean SVL difference between amplectant male and female was 2.5 mm (SD = 1.74, range -2.6–6.7, *n* = 105).

Reproductive behavior

Males began calling immediately after dusk. They called while floating in temporary ponds with water <10 cm deep. Male density at some choruses was high, resulting in some males calling a few centimeters away from each other. Males defended calling sites and aggressive interactions ensued if another male approached within a radius of <10 cm. Aggressive behavior consisted of mew-like vocalizations and attempts to clasp the rival male. Amplexus and egg deposition occurred at the same ponds where choruses were calling. Amplexus is axillary.

Operational sex ratio at choruses was male-biased. During a survey at La Maná, we recorded 14 males but only one female; at Cerro Blanco, the ratio was 3:1 (*n* = 16 individuals); at Patricia Pilar, the ratio was 8.5:1 (*n* = 19). The average ratio is 8.5:1 (*n* = 3 surveys).

Spawning behavior.—Nests were built while in amplexus, on shallow water, next to vegetation or muddy banks. The following description is based on an amplectant pair found on 04 February 2004 at ~1:00 AM (male QCAZ 26672, SVL = 26.2 mm, hereafter referred as α-male; female QCAZ 26671, SVL = 31.7 mm) building a foam nest. At the beginning of the observation, the nest already had a diameter >50 mm. The male remained in amplexus until the couple left the nest (50 minutes later). To form the foam, the male kicked the egg mass while they were being extruded from the female's vent. Kicking occurred in regular bursts with intervening periods during which the couple was immobile. In a typical burst cycle, the male's legs move downward, presumably to place his feet next to the female vent. Then, the male's feet move up until they reach the posterior end of his dorsum. At that moment, usually one or two eggs become visible in the jelly matrix between the feet. This is followed by a series of ~20 rapid kicks on which his legs become partly extended backward and then distended forward until reaching the posterior end of his dorsum. During these kicks, his legs move simultaneously but in opposite directions (forward-backward) and feet momentarily touch medially. The burst ends with to 2–4 forceful kicks on which his legs are nearly completely extended posterolaterally, partly removing the foam that lies immediately behind the couple. Each male burst seems to be triggered by an abdominal movement of the female.

Each burst of kicking lasted on average 4.64 s (SD = 0.53, range 2.13–6.22, *n* = 215); the intervening immobile periods lasted 9.25 s (SD = 12.15, range 0.12–119, *n* = 215). Total duration of bursts was 16'30" during 50' of observation. The duration of each burst and the number of bursts decreased during the second half of the sequence (Fig. 3).

Multimale nesting behavior.—Multimale spawning was only observed once, during the spawning event described in the previous section (male QCAZ 26672, female QCAZ 26671). From the beginning of the observation, a peripheral adult male (QCAZ 26673; hereafter referred as β-male) was sitting on the nest edge, directly opposite to the nesting couple and with the posterior ½ of its body embedded in the foam (Fig. 4). On at least five occasions its body moved slightly from side to side in sequences that lasted 3–4 s (Fig. 3). The movements were always in concert with the kicking bursts of the α-male.

Table 1. Pearson's correlation coefficients and ANOVA's *P* values for linear regressions. Body condition is defined as the residuals between SVL and mass. SVL = snout-vent length.

Variable 1	Variable 2	R^2	*n*	*P*
Nest volume	Female size (SVL)	0.082	74	0.013*
Nest volume	Female mass (before oviposition)	0.020	56	0.287
Nest volume	Male size (SVL)	0.026	74	0.168
Nest volume	Male condition	<0.001	74	0.796
Nest volume	Number of eggs	0.241	70	<0.001*
Number of eggs	Male size (SVL)	0.051	76	0.049*
Number of eggs	Female size (SVL)	0.027	76	0.151
Number of eggs	Female mass (before oviposition)	0.111	62	0.008*
Number of eggs	Female mass (after oviposition)	0.128	58	0.006*
Number of eggs	Female condition	0.125	62	0.005*
Number of eggs	Male condition	0.011	76	0.352

*Significant at $P < 0.05$

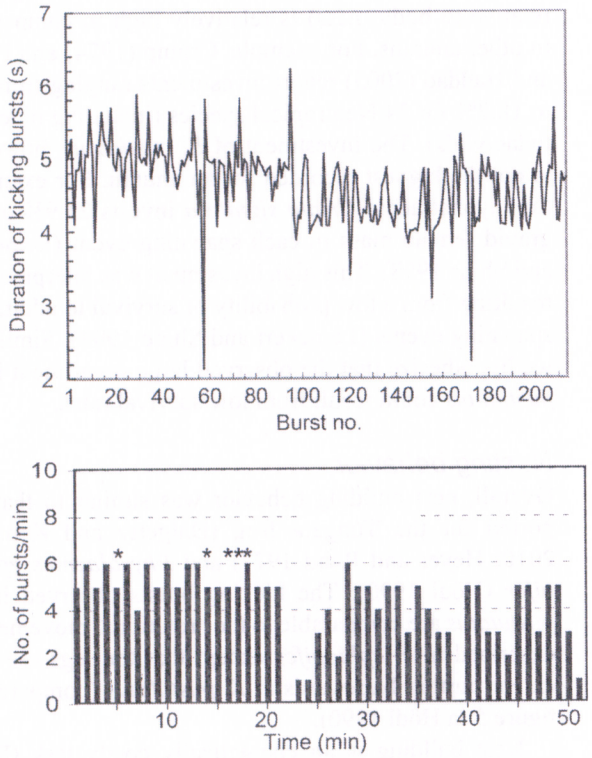


Fig. 3. Spawning of *Engystomops pustulatus* nesting couple (QCAZ 26671–72) and β-male (QCAZ 26673). Above: duration of kicking bursts. Below: number of bursts per minute; asterisks indicate β-male movements in the foam. Measurements are shown in sequence from the beginning of the observation until the couple left the nest. See text for details.

Most likely, the movements were generated by kicking bursts of the β-male legs (hidden below the foam). He left 23 min later, apparently following an amplectant couple (not collected) that approached at a distance of 10 cm from the nest (see below).

The β-male (SVL = 25.3 mm) was one of the smallest in the population. Out of 49 calling males measured during the same season, only three were smaller (mean SVL = 27.55 mm, SD = 1.23); out of 59 males found in amplexus, only one was smaller (mean SVL = 27.9 mm, SD = 1.20). Assuming a normal SVL distribution, the probability of drawing a male with equal or lower SVL by chance is 0.020 (*z*-score = –2.058). On a sample of seven males including the β-male, mean testes mass was 0.47% of total body mass (range 0.24–0.70%; mean body mass = 1.59 g, SD = 0.28). Contrary to our expectations, the β-male had the proportionally smallest testes.

Discussion

Clutch size, fertilization success, and parental investment

Number of eggs/clutch in *Engystomops pustulatus* is ~37% higher than in the túngara frog (Ryan 1985). In several anurans, clutch size is significantly correlated to body size (e.g., Crump 1974; Ryan 1985; Wells 2007). In *E. pustulatus*, such a relationship was significant for female condition and gravid and non-gravid female mass. However, the relationship was not significant for female SVL. Interestingly, we also found a significant correlation between number of eggs and male SVL suggesting that larger males have a higher reproductive success. This correlation could not be explained by indirect correlations with the other measured variables because they were either uncorrelated with male SVL (e.g., nest volume) or uncorrelated with number of eggs (e.g., female SVL).

Female SVL and number of eggs are correlated with nest volume. Nest foam also results from intense male physical activity. However, we were unable to find a relationship between nest volume and either male size or male body condition (Table 1).

We found size assortative mating as large females have a tendency to mate with large males. An adaptive explanation for size assortative mating states that it increases fertilization rates because it results in female and male vents being closer during amplexus (Licht 1976). Evidence for this scenario has been reported for the Túngara frog (Ryan 1985). *Engystomops pustulatus* lacks

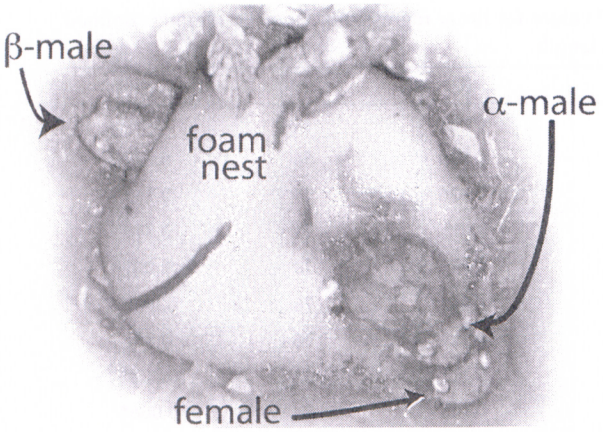


Fig. 4. *Engystomops pustulatus* nesting couple (QCAZ 26671–72) and β-male (QCAZ 26673). The couple builds the foam nest as the male kicks the egg masses extruded by the female. Frame from video (infra-red recording). See text for details.

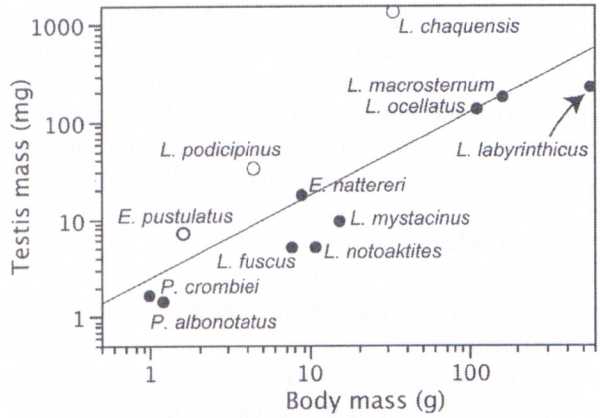


Fig. 5. Relationship (in log space) for body and testis mass among 11 species of Leptodactylinae frogs. Except for *Engystomops pustulatus*, data is from Prado and Haddad (2003). Open circles indicate species on which multimale spawning has been reported. Note that *E. pustulatus*, in which multi-male spawning apparently occurs, also has larger testis than other Leptodactylinae.

that relationship as demonstrated by couples with large differences in size (2–4 mm) showing high fertilization rates (Fig. 1A). The lack of influence of size difference on fertilization may be explained by our observation of spawning behavior because the male uses his feet to drag the eggs from the female's underside to his own vent. Therefore, the relative position of male and female vents may have a minor influence in the relative position of eggs and released sperm. Fertilization rates are generally high (more than 98% on average) suggesting that size differences between male and female have little influence in individual fitness. Similar results have been reported in other explosive breeding anurans like *Lithobates sylvaticus* (Howard and Kluge 1985) and Anaxyrus cognatus (Krupa 1988).

Size assortative mating could also result from non-adaptive interactions. If small males mating with large females are more easily displaced than large males mating with large females, a size correlation will result (e.g., Howard and Kluge 1985). This mechanism seems unlikely in *Engystomops*. During our fieldwork with *E. pustulatus* and with other species of *Engystomops* in the Chocó and the Amazon region, we never saw unmated males attempting to displace amplectant males. Attempts were rare in *E. pustulosus* and all of them were unsuccessful (Ryan 1985). Therefore, an explanation for size assortative mating in *E. pustulatus* and its sister species, *E. puyango* (reported by Ron et al. 2010) is pending.

Reproductive investment (or effort) is a measure of the allocation of energy in reproduction relative to total energy (Pianka 2011). Theory predicts that a high reproductive investment should be more adaptive if females are unlikely to survive to another reproduction event (Williams 1966). Our estimate of mean reproductive investment for *Engystomops pustulatus* (15.2%; egg mass relative to body mass) is relatively high in comparison to other anurans. For example, Crump (1974) and Prado and Haddad (2005) report investments ranging from 3.1 to 18.2% for 34 Neotropical species (including nine leptodactylids). The investment of *E. pustulatus*, however, is not the highest recorded for an anuran. For example, the myobatrachid *Crinia signifera* invests 25.9% of the gravid female mass in each spawning event (Lemckert and Shine 1993). This high investment was interpreted as resulting from a low probability of survival to additional spawning events (Lemckert and Shine 1993). Similarly, we hypothesize that the observed large investment in *E. pustulatus* could result from low survival rates.

Nesting behavior

Overall, nest building behavior was similar to that reported for the Túngara frog (Dalgetty and Kennedy 2010; Heyer and Rand 1977) and *Physalaemus ephippifer* (Hödl 1990). The kicking bursts observed in *E. pustulatus* are comparable to the "rotational movements" described in *P. ephippifer* except that the legs seem to extend further backwards in *E. pustulatus* (compared to figure 5 in Hödl 1990).

Nest building is an energetically costly task (Ryan 1985) and the observed decrease in the frequency of kicking bursts towards the end of spawning (Fig. 3) was also reported in the túngara frog (Ryan 1985) and *Physalaemus ephippifer* (Hödl 1990). As in *Leptodactylus labyrinthicus*, the Túngara frog, and *P. ephippifer*, kicking bursts seemed to be triggered by a female abdominal movement (Heyer and Rand 1977; Hödl 1990; Silva et al. 2005). The movie quality did not allow us to determine whether the decrease in burst frequency was male or female-driven in *E. pustulatus*.

Multimale mating behavior

Our observation of more than one male spawning with a female during oviposition suggests that multiple paternity and alternative reproductive strategies may exist in

Engystomops pustulatus. Although the β-male was not in amplexus, its movements were similar and in synchrony with those of the amplectant male, suggesting that it was attempting to fertilize eggs (clutch piracy). A similar reproductive behavior (with synchronic leg movements) has been reported in *Leptodactylus chaquensis* although with up to seven males in addition to the amplectant male (Prado and Haddad 2003). Egg fertilization by peripheral non-amplectant males has also been demonstrated in *Chiromantis xerampelina*, a foam-nesting rhacophorid (Jennions and Passmore 1993).

The evolution of multimale spawning should be facilitated in reproductive systems where: (1) the operational sex ratio is strongly male biased, (2) fertilization is external, (3) fecundity is high, and (4) eggs are spatially aggregated (Byrne and Roberts 2004; Shuster and Wade 2003). All this characteristics are part of the reproduction of *E. pustulatus*. Therefore, the occurrence of multimale spawning was probable. As previously reported in the Túngara frog (Ryan 1983), our data suggests that an individual male is frequently unable to fertilize all the eggs of a clutch, even in the absence of sperm competition. Although the presence of unfertilized eggs suggests the potential for fitness gain of a β-male sneaking into the nest of an amplectant pair, the proportion of unfertilized eggs was typically low (1.89% on average). Higher fitness gains for the β-male may result from sperm competition.

We could not determine the frequency of multimale spawning in the population. We observed monoandrous spawning frequently and multimale spawning was only recorded once, suggesting that it is relatively infrequent. This is consistent with observations across a variety of taxa showing that β-male strategies exist at a low frequency in natural populations (Shuster and Wade 2003; but see Byrne 2002; Jennions and Passmore 1993). The low number of reports of multimale spawning among leptodactylids is surprising because the characteristics of the reproductive system of Leptodactylidae should favor the evolution of secondary male mating strategies. The paucity of known cases may be, at least partly, a sampling artifact because the reproductive behavior has been described in only few species.

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