PRESENCE AND ABSENCE OF THE CEMENT GLAND IN FOAM-NESTING LEPTODACTYLIDS (ANURA: LEPTODACTYLIDAE): IMPLICATIONS FOR THE TRANSITION TO TERRESTRIAL DEVELOPMENT

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Reproduction and early development are compared in three foam-nesting leptodactylids: *Leptodactylus fuscus*, *L. validus* and *Physalaemus pustulosus*. *Physalaemus pustulosus* and *L. validus* produce floating foam nests containing large numbers of small eggs which hatch early and soon leave the nest as larvae, attaching to solid surfaces by their cement glands until the stage of independent feeding. *Leptodactylus fuscus* foam nests are deposited in terrestrial burrows and contain small numbers of large eggs which hatch early, but remain in the foam nest until the yolk is resorbed and the larva has developed its tail for effective locomotion. The lack of a cement gland in *L. fuscus* suggests that the post-hatching period in the nest is a normal part of development. *L. fuscus* eggs and hatchlings transferred prematurely to water displayed low survival. The results are discussed in the context of evolutionary reproductive transitions within the leptodactylids.

Key words: cement gland, foam-nest, frog reproductive strategy, Trinidad

INTRODUCTION

The cement gland is a transient embryonic and early larval structure in anuran amphibians, first seen before hatching at around Gosner (1960) stage 14-15 and usually disappearing around the onset of feeding, at stage 25-26. Its role is to produce a sticky secretion that allows newly hatched larvae to hang motionless from egg capsules, water surface films or structures in water such as plant stems and rocks: during this period, external gills regress and mouthparts develop until the larva is ready to feed (Sive & Bradley, 1996).

However, not all anurans have a cement gland. Duellman & Trueb (1986) noted that embryos with prolonged intra-capsular development and a late hatching stage may have transient cement glands, and that direct developing species, such as the eleutherodactylids, may lack them altogether. Thibaudeau & Altig (1999) expected that cement gland loss should be a general feature in endotrophic anurans – i.e. those that develop the adult form without feeding on external sources – and reported that studies so far have shown an absence of cement glands in endotrophic species not developing in a nest (such as viviparous and marsupium-brooded species) and that cement glands are often not functional in nest-laying endotrophs.

Cement gland presence or absence and morphology are rather rarely reported in general accounts of anuran characteristics, probably because of the gland's transience, but it is clearly a key feature in evolutionary transitions from aquatic to non-aquatic modes of reproduction. In a study of comparative development and morphology of the cement gland in 20 anuran species from six families, Nokhbatolfoghahai & Downie (2005) found that the cement gland was present and functional in three species of foam-nesting leptodactylids (*Physalaemus pustulosus*, *Leptodactylus validus* and *L. bolivianus*) but absent in the fourth, *Leptodactylus fuscus* and in the direct-developing *Eleutherodactylus urichi*. Absence in the direct developer was expected, but absence in *L. fuscus* was more surprising. *Leptodactylus fuscus* deposits eggs in a mass of foam in a burrow near a temporary pool site. After hatching, the larvae can remain in the foam nest and produce a new kind of foam which replaces the original nest foam. If heavy rains do not fall, the larvae remain in the nest in a state of arrested development for up to several weeks until rain eventually washes them into the nearby pool, where the larvae begin to feed (Downie, 1984, 1994). Nokhbatolfoghahai & Downie (2005) suggested that the absence of cement glands in *L. fuscus* may be because it is obligatory for *L. fuscus* larvae to remain in the nest until past the phase when the cement gland is functional in other species, i.e. until the stage of independent feeding. The work reported here is an exploration of this hypothesis, using a comparison of reproductive strategies amongst three foam-nesting species exhibiting different reproductive modes.

MATERIALS AND METHODS

Spawn of three leptodactylid species (*Leptodactylus fuscus, L.validus* and *Physalaemus pustulosus*) was collected from various locations in Trinidad, West Indies, over several field seasons. Spawn characteristics are described by Kenny (1969). Spawning sites were identified from adult calls at night and spawn collected

the next morning. *Leptodactylus fuscus* spawn is hidden in burrows on the banks of temporary pools, and we located burrows by probing with a metal spoon handle. *Leptodactylus validus* spawn is located at the edges of pools, usually in forest, and hidden by dead leaves: we located spawn by systematic searching of the margins of pools. *Physalaemus pustulosus* spawn is easily located floating on the surface of pools in the open. After collection, spawns were incubated in a laboratory at 27-28°C air temperature either floating on the surface of dechlorinated tap water (*L. validus, P. pustulosus*) or in a closed container on the surface of damp tissue paper (*L. fuscus*) until appropriate stages were reached (Gosner, 1960, staging used for all species). Clutch sizes were counted from the number of hatchlings, and also by checking foam nests for undeveloped eggs. Specimens for microscopic examination were fixed in buffered neutral formalin or 2.5% glutaraldehyde in phosphate buffer. Measurements of fixed embryos were made using a dissecting microscope with calibrated eyepiece graticle, and drawings were made with the aid of a drawing tube.

To test how well embryos developed if they entered water at an earlier stage than normal, eggs were carefully removed singly, using forceps, from several foam nests and floated on the surface of dechlorinated, aerated tapwater in the laboratory. For *L. fuscus*, embryos were also removed from their foam nests and placed in groups of 10 on the surface of damp tissue in 9 cm diameter Petri dishes. Success of development was monitored daily over several days.

RESULTS

Tables 1 and 2 show reproductive and developmental data for the three species, while Fig. 1 shows camera lucida drawings of embryo and larval morphology up to stage 25-26 and measurements of relative tail length. *Leptodactylus fuscus* produces small numbers of large eggs; *L. validus* large numbers of small eggs; *P. pustulosus* is intermediate. Correcting for body size, *L. validus* puts the most resource into each clutch (Table 1), *L. fuscus* the least (assuming that resource input per egg is proportional to egg volume). *L. validus* and *P.*
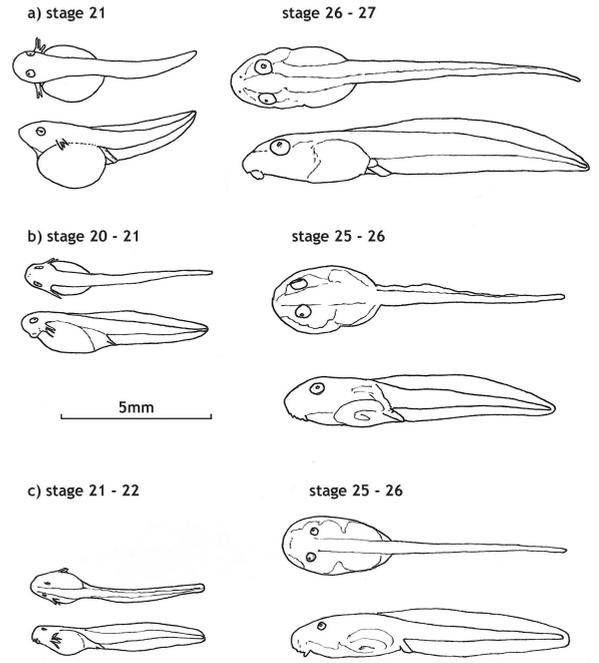
FIG. 1. Camera lucida drawings comparing early larval morphology of (a) *Leptodactylus fuscus*, (b) *Physalaemus pustulosus* and (c) *Leptodactylus validus*. Drawings made at overall magnification of x9.4. Each stage shown from dorsal and lateral aspects. All from formol-saline fixed specimens. Ratio of tail length (from middle of vent) to total body length, based on mean of three specimens is: *L. fuscus* stage 21, 50.2%; stage 26-27, 59.7%. *P. pustulosus* stage 21, 54.9%; stage 25-26 58.3%. *L. validus* stage 21-22 62.7%; stage 25-26, 59.0%.

pustulosus hatch at about the same stage and time since deposition, and leave the nest for the water below soon after (Downie, 1993 showed that some *P. pustulosus* remain in the nest for up to about 20 hr after hatching, but others leave much sooner. Comparable observations have not been made on *L. validus*). By the time they leave the nest, the larvae of these two species have greatly reduced yolk sacs and well developed tails. *Physalaemus pustulosus* larvae attach by their cement glands to the sides or bottom of their container; *L. validus* mainly hang from the lower side of their foam nest.

TABLE 1. Reproductive data for three leptodactylids. Female snout-vent length (SVL) from Murphy (1997). Egg volume calculated from diameter using formula for a sphere. 1, clutch sizes counted to nearest 100. Size related reproductive output is mean number of eggs per clutch times egg volume divided by female SVL.

Species and female SVL	Clutch size mean ±SD, n	Egg diameter (mm): mean±SD, n	Mean egg volume	Size related reproductive output
Leptodactylus fuscus 50 mm	154±38 n=10	1.97±0.17 n=9	4.0	12.3
Physalaemus pustulosus 32 mm	420±135 n=13	1.51±0.06 n=11	1.8	23.6
Leptodactylus validus 51 mm	1500±400[1] n=9	1.36±0.07 n=11	1.3	38.2

TABLE 2 . Developmental data for three leptodactylids. All species incubated at 27-28°C. 1, timing assumes eggs were fertilized in the middle of the night prior to the morning of collection.

Species and stage of hatching	Time to hatching (days)[1]	Time to stage 25 (days)[1]	Total length, mm (mean±SD, n) at different stages		
			22	23	25
Leptodactylus fuscus stage 18-19	2.5	4.5	5.7±0.1 $n=7$	6.8±0.2 $n=7$	8.4±0.2 $n=9$
Physalaemus pustulosus stage 20-21	2.0	3.5	6.8±0.3 $n=4$	7.6±0.2 $n=8$	8.3±0.2 $n=6$
Leptodactylus validus stage 20-21	2.5	3.5	5.9±0.3 $n=8$	-	7.3±0.7 $n=8$

In contrast, *L. fuscus* hatches slightly earlier in terms of developmental stage than the other two species; at comparable stages up to stage 25, they have larger yolk sacs and more poorly developed tails. Overall, development to the stage of independent feeding takes longer than in the other two species. Body length at stage 22 is shorter in *L. fuscus* than the other two species, but longest by stage 25.

Table 3 shows the results of experiments where *L. fuscus* embryos were transferred singly either to the surface of water or to damp tissue at different developmental stages. In neither case was development very successful until about stage 22 (day 3, when embryos were already hatched). Mortality was high in both cases for embryos transferred during the first two days of development. However, the pattern of mortality was different in the two cases. Early embryos transferred to the water surface floated until hatching, then sank to the bottom. Many failed to reach hatching stage and those that did hatch often appeared malformed and soon died. Early embryos transferred to damp tissue tended to develop successfully past the hatching stage and died between days 3 and 4: it looked as if the lack of support for the large yolk mass when the embryo was outside the foam nest had some kind of harmful effect, possibly blocking the yolk sac circulation. Embryos transferred to the damp tissue surface at a later stage survived and developed well.

When a similar experiment was carried out on *P. pustulosus*, all embryos either on water or tissue surface developed normally to hatching. Those in water all continued to develop normally; those on damp tissue died soon after hatching. No *L. validus* embryos were available for comparable experiments.

DISCUSSION

Our conclusion from the observations reported here is that *P. pustulosus* develops like most anurans, except in a floating foam nest. Once hatched, with a reduced yolk mass, larvae enter water and attach to a surface by their cement gland until the external gills are resorbed and feeding can begin. Tails are well developed at an early stage to allow active swimming. Out of water, hatchlings have very brief survival capacity, a feature noted by Downie (1988) and also for later stage tadpoles by Downie & Smith (2003). We expect that *L. validus* hatchlings would also show poor survival out of water, but have not tested them. *Leptodactylus fuscus* shows several differences from the *P. pustulosus* reproductive mode: the foam nest is in a terrestrial burrow, and eggs are both larger and in relatively smaller numbers. Development to the stage of independent feeding takes longer and the tail is relatively poorly developed at early stages. Downie (1994) has shown that after hatching, *L. fuscus* larvae make a foam which replaces the original nest foam and can remain in the nest for up to several

TABLE 3. Survival of *L. fuscus* and *P. pustulosus* eggs transferred at different stages to the surface of water or damp tissue paper.

Stage on transfer	Percentage of survival to stage 26 (*L. fuscus*, day 6; *P. pustulosus,* day 4); n=number of eggs at start; *L. fuscus* pooled from several clutches, *P. pustulosus* from one clutch.	
	Transfer to water	Transfer to tissue
L. fuscus		
Cleavage (day 0)	0 ($n=12$)	10.0 ($n=20$)
Gastrula (day 1)	39.6 ($n=48$)	33.3 ($n=30$)
Hatching (day 2)	56.0 ($n=50$)	0 ($n=34$)
Stage 22 (day 3)	100.0 ($n=50$)	50.0 ($n=10$)
Stage 23 (day 4)	100.0 ($n=31$)	100.0 ($n=10$)
P. pustulosus		
Cleavage(day 0)	100.0 ($n=20$)	0 ($n=20$)

weeks, depending on rainfall. The results reported here suggest that at least a brief post-hatching period in the foam nest is necessary for effective development. Early entry to water leads to high mortality and early hatch-lings do not survive well on a damp tissue surface, though they do at a somewhat later stage (Downie & Smith, 2003). Poor early survival either in water or on damp tissue may be related to the large size of the yolk mass and the relatively poorly developed locomotory organs. To improve survival chances, the larvae remain in the foam nest till the yolk mass is reduced and the tail fully developed for locomotion. This takes the larvae beyond the stage when a cement gland is normally functional. As has been repeatedly demonstrated, natural selection leads to the reduction and eventual deletion of functionless organs (Fong *et al*., 1995). Nokhbatol-foghahai & Downie's (2005) finding of vestiges of cement gland development in *L. fuscus* suggests that this deletion is fairly recent. We would expect the cement gland to be lacking in other leptodactylids that spend a post-hatching period in the nest, even if they have a tad-pole stage, such as other species in the *Leptodactylus* '*fuscus*' group (Heyer, 1969).

It is not clear why *L. fuscus* should hatch relatively early, if the embryos must then spend a further period in the nest. In other frogs with large eggs and later emer-gence to water, such as the myobatrachid *Pseudophryne bibroni* (Bradford & Seymour, 1985) or members of the genus *Phyllomedusa* (Pyburn, 1980), hatching is after stage 25. A consequence of early hatching in *L. fuscus* is the breakdown of the original nest foam (Downie, un-published observations) which is then replaced by the new foam made by the larvae (Downie, 1984). How-ever, it is not obvious that this is an advantage, unless it is easier for the larvae to extricate themselves from the new foam, once the nests are inundated, than it would be from the original foam. Another possibility is that hatch-ing allows the larvae to escape from nest predators such as frogfly maggots (Downie *et al.*, 1995), but their poor motility immediately after hatching makes this unlikely.

Heyer (1969) outlined a possible adaptive scenario for the reproductive modes found in the genus *Leptodactylus*. Floating foam nests, as found in the '*melanonotus*' and '*ocellatus*' species groups evolved as a means of avoiding aquatic predators and the desic-cation of temporary pools; nests in potholes near water in the '*pentadactylus*' group gave further protection from aquatic predators; nests in terrestrial burrows such as in the '*fuscus*' group, removed the linkage of repro-duction and rainfall and gave hatchlings a head-start over competitor species; finally, in the '*marmoratus*' group, later moved to the genus *Adenomera* (Heyer, 1974), large eggs developed through to metamorphosis in a terrestrial egg chamber, removing the link to the aquatic habitat. More recently, Prado *et al.* (2002) ex-panded Heyer's list by two extra modes: nest hole excavation in *L. podicipinus* and larval development in the foam nest within a burrow in some species of the

'*pentadactylus*' group: Prado *et al.*'s conjecture that the latter mode might involve an extra source of nutrition, has been confirmed by Gibson & Buley (2004) for *L. fallax* where the female provides repeated batches of trophic eggs. We expect that *L. fallax* will lack the ce-ment gland.

The question remains whether or not these different modes represent a single trend towards terrestrial devel-opment. Heyer (1969) considered that the '*fuscus*' and '*marmoratus*' groups represented two separate shifts from an original aquatic foam nest. In our view, more detailed work of the kind we report needs to be done to tease out the fine details of the changes involved in these various lineages. There are several differences between the reproductive strategy and associated features of *L. fuscus* and the two floating foam nesting species we have used for comparison: these include egg size, egg number, reproductive effort, egg location, developmen-tal time out of water, survival and cement gland presence. An additional factor requiring investigation is the inter-clutch interval for the females of foam-nesting species. Davidson & Hough (1969) found that females of *Physalaemus* (=*Engystomops*) *pustulosus*, could ovu-late every 4-5 weeks in a laboratory population, but equivalent field data are lacking. It is not clear which of these differences are likely to have been primary, and which are consequences of the shift to terrestrial repro-duction. A robust phylogeny of the leptodactylids would be a great help in attempting to explain these evolution-ary changes. In addition, we need reproductive strategy data from more species, including the state of the cement gland.

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