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Reproduction in Brevicipitid Frogs (Amphibia: Anura: Brevicipitidae)—Evidence from *Probreviceps m. macrodactylus*

HENDRIK MÜLLER, SIMON P. LOADER, WILIRK NGALASON, KIM M. HOWELL, AND DAVID J. GOWER

An egg clutch of the Tanzanian brevicipitid frog, *Probreviceps m. macrodactylus*, is described for the first time. The single clutch consists of 32 eggs and 21 infertile, jelly-filled capsules, tightly bound together by a thin, tough outer layer. The infertile capsules are concentrated toward the top of the clutch. The eggs were found in a dry season, and were perhaps likely to hatch at the onset of the short rains. The specific identification is confirmed through analysis of mitochondrial DNA sequence data. Eggs are yolky and large, about 8 mm in diameter (including capsule), suggesting that *P. m. macrodactylus* has a direct mode of development. The embryos observed were in an early developmental stage and showed the early division of the brain and anlagen of three pairs of visceral arches. Brevicipitid reproduction and development are poorly known, but a review of the existing literature and parsimonious optimization of simple characters onto a phylogenetic framework suggest that direct development is a derived condition characterizing the whole clade.

RECENT years have seen many advances in understanding of the systematics of African frogs (Loader et al., 2004; van der Meijden et al., 2005; Frost et al., 2006). However, for many species, knowledge of life history is limited, and for some even the most basic biological data are lacking. One group for which life history data are scant is the Brevicipitidae (=Brevicipitinae of some authors), a small but well-defined clade (Loader et al., 2004) that ranges from South Africa through East Africa to the Bale Mountains of Ethiopia. Brevicipitids were long considered to be a subfamily of the cosmopolitan Microhylidae, but recent morphological (Blommers-Schlösser, 1993; Wu, 1994) and molecular studies (Darst and Cannatella, 2004; Loader et al., 2004; Frost et al., 2006) have revealed that they are instead most closely related to the sub-Saharan Hemisotidae. Loader et al. (2004) and Frost et al. (2006) have recently used DNA sequence data to investigate phylogenetic relationships within Brevicipitidae.

Although some species might be partly arboreal at times (de Sá et al., 2004), adult brevicipitids are typically cryptic and spend extended periods of time in soil and/or leaf litter (Barbour and Loveridge, 1928; Poynton and Broadley, 1967; Minter, 2004a). This habit probably accounts, in part, for the paucity of life history information. Most knowledge of brevicipitid life history stems from observations of species of the most speciose genus, the southern African *Breviceps.* Available data show that these frogs lay terrestrial eggs in an underground chamber that develop directly into froglets without an aquatic tadpole stage (de Villiers, 1929; Wager, 1965). So far, very little or nothing is known about the breeding biology of the other brevicipitid genera *Balebreviceps, Callulina, Probreviceps,* and *Spelaeophryne.* We describe here for the first time an egg clutch and an early embryonic stage of *Probreviceps m. macrodactylus* from the East Usambara Mountains, Tanzania. We discuss the implications of these data for understanding life history evolution in brevicipitids and their close relatives.

MATERIALS AND METHODS

A clutch of amphibian eggs was collected from Kwamkoro Forest, Amani Nature Reserve, Muheza District, Tanga Region, Tanzania, by staff working for Frontier–Tanzania. The locality (5°7′S, 38°38′E, 950 m asl) is in the East Usambara Mountains of the Eastern Arc. The eggs are preserved in 70% IMS, catalogued as BMNH 2000.775 in the collections of The Natural History Museum, London. There are no field notes associated with the eggs, but they were part of a mixed batch of amphibian specimens collected between 13 July and 21 August 1999. The eggs were thus found in the longer of two drier seasons in Amani, between the March–May and November rainy seasons (Fig. 1). In

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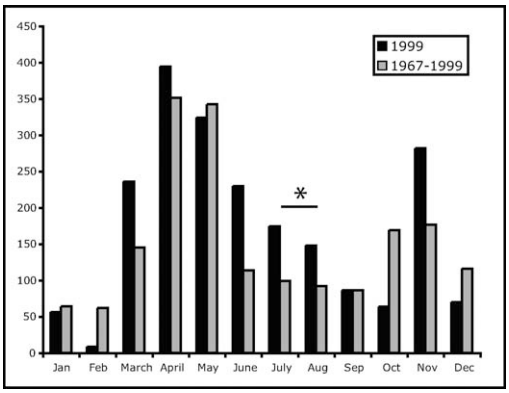


Fig. 1. Histogram showing monthly rainfall data for 1999 and averaged for 1967–1999 in Amani. *Indicates the collection date for the *Probreviceps m. macrodactylus* egg clutch.

order to confirm the provenance of the egg clutch, a single embryo was removed and DNA was extracted using a Qiagen DNeasy kit. One partial fragment each of the mitochondrial 12S and 16S mitochondrial genes were amplified and sequenced using methods reported in Loader et al. (2004). Both gene sequences were subjected to a BLAST search via GenBank. The concatenated sequences were added to a previous brevicipitid alignment (Loader et al., 2006) and subjected to parsimony and maximum likelihood phylogenetic analyses. Parameter settings for all phylogenetic analyses were calculated by Modeltest 3.04 (Posada and Crandall, 1998), with the GTR I + G model selected. For maximum likelihood analyses, the tree bisection–reconnection branch swapping (with ten random-addition sequence replicates) heuristic search option was used. A branch and bound search option was used for parsimony analysis. Support for clades was measured with bootstrap proportions of 10,000 pseudoreplicates for parsimony and 1,000 for maximum likelihood (Felsenstein, 1985). Trees were rooted using the microhylid *Hoplophryne uluguruensis* as an outgroup (Loader et al., 2004). MacClade (vers.4.08, D. R. Maddison and W. P. Maddison, MacClade 4: analysis of phylogeny and character evolution, Sinauer Associates, Sunderland, MA, 2005) was used to establish most parsimonious optimizations of characters on phylogenies.

From the clutch, the diameters of ten fertile eggs and ten empty capsules were measured. For observation of developmental stages, outer capsules were removed from four eggs using sharpened watchmaker's forceps, and the gelatinous layer gently removed by rolling the egg onto absorbent tissue paper. Measurements were made using a ruler or under a microscope with an ocular micrometer. Photographs were taken with a digital camera (Nikon Coolpix 995) attached to a Nikon SMZ-U stereomicroscope.

Results

Identity of eggs.—For the partial 12S sequence of the eggs (GenBank Accession DQ457065), the BLAST search found a 100% match to an East Usambara *Probreviceps m. macrodactylus* (voucher KMH 21399, Loader et al., 2004). For the 16S sequence (DQ457066) BLAST recovered a 98.42% match to a different (KMH 21461) East Usambara *P. m. macrodactylus*. Phylogenetic analysis of the 717 unambiguously aligned sites for the concatenated 12S and 16S sequences using ML and MP recovered very similar optimal trees (Fig. 2). Unsurprisingly, given the BLAST results, all phylogenetic analyses recovered the egg clutch sample as a member of a clade otherwise including only samples of adult *P. m. macrodactylus* from the East Usambara. In all other respects, the optimal phylogenetic trees are consistent with the *Probreviceps* species relationships reported by Loader et al. (2004, 2006). DNA sequence data thus strongly support the hypothesis that this is an egg clutch of *P. m. macrodactylus*.

Morphology of clutch and embryos.—The clutch (Fig. 3) had an irregular shape and was approximately 35 mm in diameter and about 17 mm high. Some small leaf fragments and other organic debris were attached to the surface of the clutch. The clutch was comprised of about 32 seemingly viable eggs and a further approximately 21 egg capsules that contained only jelly but no eggs. All eggs and infertile capsules were bonded strongly together into a single coherent clutch that was enveloped by a thin but tough outer coating. All eggs had an outer capsular layer that surrounded the gelatinous mass immediately around the egg. The whole eggs, including their capsules, ranged in diameter from 7 mm to 8.82 mm (n = 10, mean = 7.95 mm, SD = 0.63). Eggs removed from their capsules were not perfectly spherical, but slightly compressed along the animal–vegetal axis, and ranged in size from 4.20 mm × 3.92 mm to 5.04 mm × 4.48 mm. Infertile capsules lacking eggs were more variable in size, with diameters ranging from 1.96 mm to 4.62 mm (n = 10, mean = 3.23, SD = 0.98). These infertile capsules were concentrated on what we interpret to be the upper side of the clutch, based on the orientation of the embryos inside the eggs, with far fewer present on the underside.

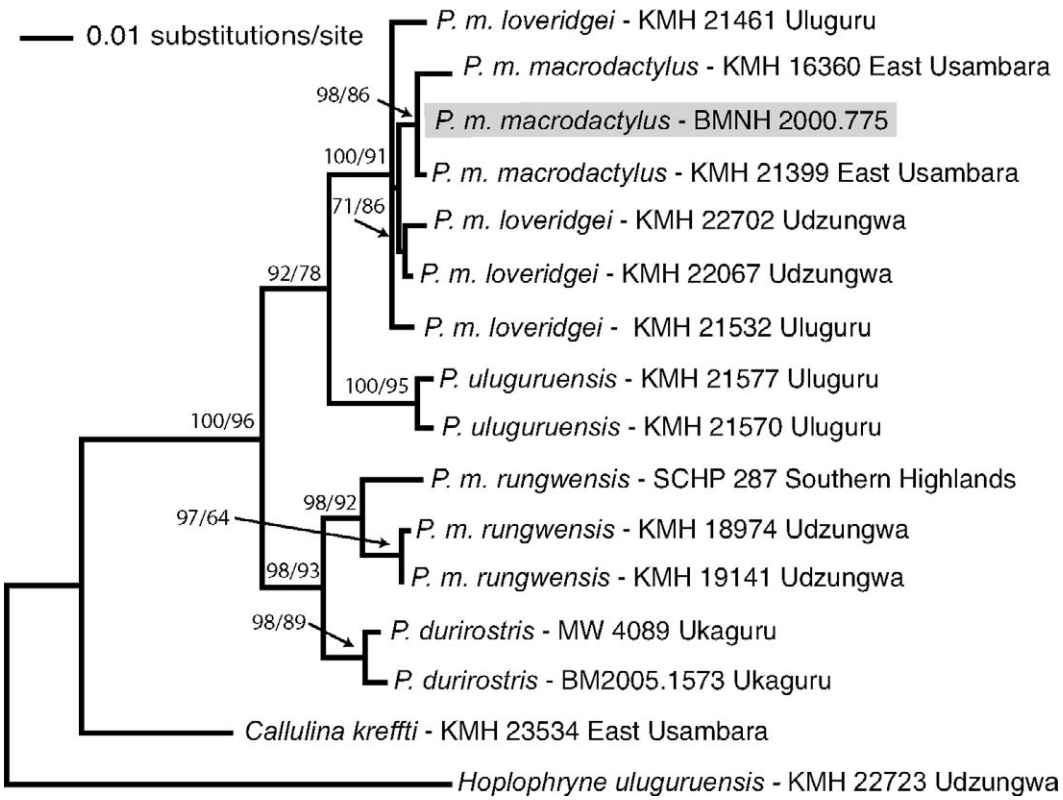


Fig. 2. Maximum likelihood phylogeny (–ln likelihood = 2502.31362) of *Probreviceps* species from analysis of partial mitochondrial 12S and 16S DNA sequences. Heuristic search settings (as estimated using Modeltest 3.04 by Posada and Crandall, 1998) are: Proportion of invariant sites = 0.3825, Gamma shape parameter = 0.6648, 4 rate categories, Nucleotide frequencies A = 0.31790, C = 0.24320, G = 0.19780, T = 0.3825. Values above branches correspond to Parsimony Bootstrap results (10,000 replicates) and Maximum Likelihood Bootstrap (1,000 replicates).

All eggs were pale and unpigmented and had a light yellow color in preservative. The four eggs removed from their envelopes were at a similar, early stage of development. Unfortunately, the preservation of the eggs was not ideal, and the embryos appeared somewhat dehydrated, which made it more difficult to distinguish structures. The head region was well differentiated. The brain was divided into fore-, mid-, and hindbrain, and the forebrain had conspicuous lateral swellings corresponding to the telencephalic lobes. Anterior to the forebrain was a small, crescent-shaped depression. Three anlagen of visceral arches were present on each side of the head region, and otic vesicles were present close to the base of the first visceral arch. About seven trunk somites were differentiated. The area surrounding the embryo was slightly darker in coloration and had a smoother appearance than the vegetal pole of the egg. Embryos resembled stage I embryos of *Breviceps mossambicus* as described by de Villiers (1929), but seemed slightly more advanced as judged by visceral arch development.

Discussion

Reproduction in brevicipitid frogs.—Although the egg clutch is not associated with an adult, and there are no microhabitat data, the egg clutch can be confidently identified as *P. m. macrodactylus* based on mitochondrial DNA sequence data. The amount of yolk supplied in the egg suggests that *P. m. macrodactylus* has a direct form of development.

Eggs and early developmental stages of brevicipitids are poorly known. Previous reports for *Probreviceps* are brief and without voucher specimens or pictures (Table 1). Barbour and Loveridge (1928) described an adult female *P. ulugurensis* found together with 20 eggs in a burrow. The eggs were cream white and measured 4 mm in diameter (5.5 mm including the capsule). Ngalason (2005) reported *P.*

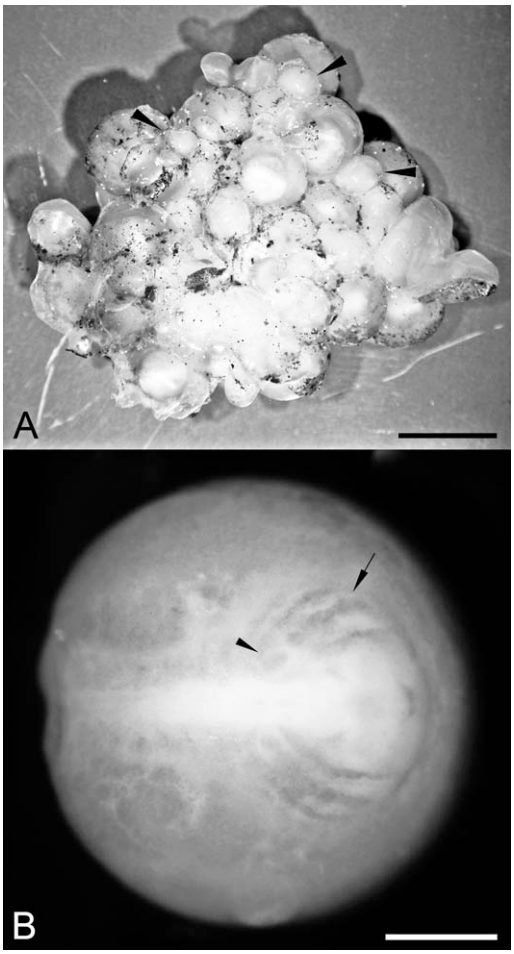


Fig. 3. Clutch (A) and embryo (B) of *Probreviceps m. macrodactylus*. Arrowheads in (A) point to some of the empty egg capsules. In (B), the arrowhead points to the otic vesicle and the arrow to the visceral arch anlagen. Scale bar in (A) equals 10 mm and 1 mm in (B).

uluguruensis to lay their eggs in burrows made beneath piles of leaf litter or beneath grass tussocks in moist soil. He further reported that *P. uluguruensis* provide parental care to the clutch and may stay with the clutch until hatching. According to his observations, *P. uluguruensis* has a non-feeding and non-swimming larval stage that develops directly inside the egg capsule. Barbour and Loveridge (1928) also mentioned the discovery of a female *P. m. loveridgei* (as *Breviceps rugosus*) in a burrow with 35 eggs, 4–5 mm in diameter (6 mm including capsules), and they further reported that this clutch contained a few opaquely white, and probably infertile, round or oblong eggs of about 1 mm in diameter. Parker (1934), likely referring to the observations of Barbour and Loveridge (1928), very briefly mentioned that *P. ulugurensis* and *P. m. loveridgei* lay their eggs in subterranean burrows and provided mean egg sizes for both species (5.5 mm and 6 mm, respectively, including capsules). Poynton and Broadley (1967) reported a female *P. rhodesianus* sitting on top of a clutch of 20 eggs, approximately 5 mm in diameter, laid inside a hollow in humus below a three-inch layer of decaying leaves. Channing and Howell (2006) briefly mentioned that *P. m. rungwensis* deposits its eggs in a burrow.

Various information on egg deposition site, clutch characteristics, and development is available for six of the 16 species of *Breviceps* (Table 1). Terrestrial development without an aquatic tadpole stage has been reported for *B. adspersus* (Minter, 2004b), *B. gibbosus* (Wager, 1965), *B. mossambicus* (FitzSimons and van Dam, 1929), and *B. sylvestris* (Wager, 1965). Other species of *Breviceps* for which data are available are also known to lay a relatively small clutch of large, yolk-rich eggs in an underground chamber. Egg clutches are covered by a layer of infertile, jelly-filled capsules, thought to provide extra moisture (Wager, 1965). Visser (1979) documented sizes of ovarian eggs for *B. montanus* (3.8 mm) and *B. vansoni* (3.12 mm) and further reported a female *B. fasciatus* with 15 (almost oviductal, J. D. Visser, pers. comm.) eggs of 3.9 mm diameter.

Probreviceps and *Breviceps* share several reproductive traits. Species of both genera produce relatively small clutches of comparatively large, yolk-rich eggs topped with several infertile egg capsules. The clutch is deposited in a secluded place, usually a subterranean burrow, and development seems to be direct in that a free-swimming tadpole stage does not occur. No observations of brooding behavior or life history have been reported for the other brevicipitid genera *Balebreviceps*, *Callulina*, and *Spelaeophryne*. Parsimony optimization of a simple, direct versus biphasic development binary character mapped onto the phylogeny reconstructs direct development for the common ancestor of *Breviceps*, *Callulina*, *Probreviceps*, and *Spelaeophryne* (Fig. 4). We further predict that all species of these four genera have direct development, including *Callulina* and *Spelaeophryne*. For the latter two genera, this is supported by reports of large (3 mm) ovarian eggs in *C. kreffti* (Barbour and Loveridge, 1928; Parker, 1934), and perhaps by ''large eggs'' in *S. methneri* (Channing and Howell, 2006). The single species of *Balebreviceps, B. hillmani*, has not been included in phylogenetic analyses to date but it is also likely to have direct development if it falls within the clade including the other brevicipitids.

TABLE 1. SUMMARY OF PUBLISHED INFORMATION ON CLUTCH AND EGG SIZES, DEPOSITION SITE, AND DEVELOPMENTAL TIME IN BREVICIPITIDAE.

Taxon	Clutch size	Egg size	Developmental time	Deposited in	Reference
Breviceps acutirostris	24	6.5–7.8 mm (mean 7 mm)	—	—	Harrison, 2004
Breviceps adspersus	About 20–46, covered by smaller mass of fluid-filled jelly capsules without yolk	4.5 mm; 7–8 mm including capsule	6 weeks	Chamber 30 cm below surface	Minter, 2004b
Breviceps fuscus	42 and 43, each topped by layer of 25–30 empty egg capsules	5 mm; 8mm including capsules	—	Chamber 30–40 cm below surface	Channing, 2001; Burger, 2004
Breviceps gibbosus	13 to 30	—	—	—	Harrison and Minter, 2004
Breviceps mossambicus	20–25	6 mm; 12 mm including capsule	6–8 weeks	Spherical chamber	FitzSimons and van Dam, 1929
Breviceps sylvestris	56 eggs covered by layer of infertile eggs	—	8 weeks	—	Wager, 1965
Probreviceps m. loveridgei	35, plus a few opaquely white eggs of 1-mm diameter	4–5 mm; 6 mm including capsules	—	Burrow	Barbour and Loveridge, 1928; Parker, 1934
Probreviceps m. macrodactylus	32, plus 21 empty capsules mostly on top	4–5 mm; appr. 8 mm including capsule	—	—	This study
Probreviceps rhodesianus	20	5 mm	—	Hollow in humus, below a 3-inch layer of dead leaves	Poynton and Broadley, 1967
Probreviceps ulugurensis	20	4 mm; 5.5 mm including capsules	—	Burrow	Barbour and Loveridge, 1928; Parker, 1934; Ngalason, 2005

Females or female–male pairs found together with eggs or froglets have been reported for *Breviceps adspersus* (Wager, 1965; Minter, 2004b), *B. gibbosus* (Rose, 1962; Wager, 1965), *B. mossambicus* (FitzSimons and van Dam, 1929), *B. sylvestris* (Wager, 1965), *Probreviceps m. loveridgei* (Barbour and Loveridge, 1928), *P. rhodesianus* (Poynton and Broadley, 1967), and *P. ulugurensis* (Barbour and Loveridge, 1928; Ngalason, 2005). The male remains with the eggs in *B. fuscus* (Channing, 2001). FitzSimons and van Dam (1929) reported that female *B. mossambicus* remain adjacent to, but outside, a sealed subterranean egg chamber, while Thompson (in Wager, 1965) observed that female *B. sylvestris* removed soil that had fallen onto the clutch during the opening of the chamber. All this suggests that some form of brood care occurs in brevicipitids, although the extent and details of this remain incompletely known.

Reproduction in the Afrobatrachia.—Based on the most recent and comprehensive analysis of frog phylogeny (Frost et al., 2006), the clade comprising Brevicipitidae and Hemisotidae (Xenosyneunitanura of Frost et al., 2006) is the sister group to the Laurentobatrachia, which comprises the traditional Hyperoliidae, Astylosternidae, and Arthroleptidae. All these groups together form an exclusively African clade of frogs, the Afrobatrachia (Frost et al., 2006). In this respect, the results of Frost et al. (2006) are consistent with previous phylogenetic hypotheses (Darst and Cannatella, 2004; Loader et al., 2004; van

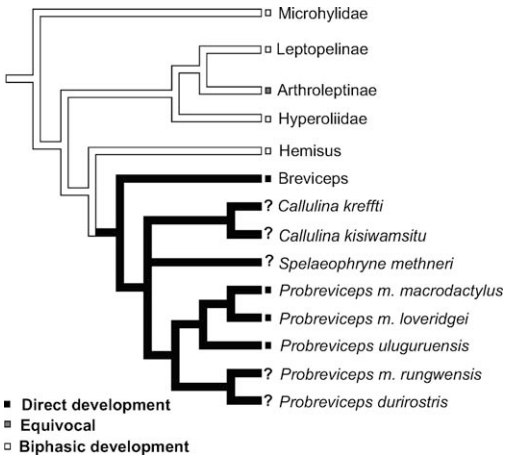


Fig. 4. Most parsimonious optimization, as reconstructed by MacClade, of the binary developmental character direct-development vs. biphasic with aquatic larvae on a phylogeny of Afrobatrachia. ? = development unknown. Framework phylogeny from current paper, Loader et al. (2004, 2006), and Frost et al. (2006). Schiøtz (1999) suggested direct-development in *L. brevirostris*, but this remains unconfirmed, and we scored Leptopelinae as having a biphasic development because all other species have free-living tadpoles, where known. However, scoring Leptopelinae as equivocal does not change the optimization shown here.

der Meijden et al., 2004). Within Afrobatrachia, direct development occurs in some (but not all) arthroleptids *sensu* Frost et al. (2006) and in brevicipitids (Guibé and Lamotte, 1958; Lamotte and Perret, 1963; Wager, 1965). Although leptopelines generally have aquatic larvae, Schiøtz (1999) suggested that *Leptopelis brevirostris* might have direct development, based on the observation of relatively large eggs (5 mm) in that species. Where known, all other afrobatrachians have free-living tadpoles but show a wide range of reproductive modes (Schiøtz, 1999; Rödel, 2000), ranging from eggs deposited in water (hyperoliines), outside water on vegetation (hyperoliines) or in shallow depressions (leptopelines), to egg deposition in a subterranean chamber with brood care and tadpole guiding (hemisotids).

Given current phylogenetic understanding, the taxonomic distribution of reproductive modes within Afrobatrachia suggests that direct development evolved independently in brevicipitids and some arthroleptids (and possibly *Leptopelis brevirostris*). Where known, females of all species of the only hemisotid genus *Hemisus* deposit and guard eggs in subterranean burrows. After hatching, females open the chamber and, if necessary, dig a slide to the nearest water body or possibly transport tadpoles on their backs (van Dijk, 1985; Rödel et al., 1995; Kamisky et al., 1999). If rainfall is insufficient at the time of hatching, tadpoles remain inside the brood chamber and can arrest their development for up to 66 days (Rödel et al., 1995). Clutch size in *H. marmoratus* ranges from 88 to 242 eggs, each 4.9 to 7.3 mm in diameter, including capsules (Rödel et al., 1995; M.-O. Rödel, pers. comm.). Rödel et al. (1995) further reported that several of the clutches found were surrounded by infertile eggs and that the clutches had a ''parchment-like'' thin but tough cover, and Rödel (2000) reported that clutches of *H. marmoratus* are usually covered by a layer of infertile eggs. In these respects, hemisotid clutches resemble those of brevicipitids.

Although data are incomplete, it seems that *Hemisus* and brevicipitids share the derived reproductive characters of large to moderately large eggs, infertile egg capsules on the outside of the clutch, deposition in terrestrial subterranean burrows, and probably brood care. Based on outgroup comparison, the terrestrial eggs, parental care, and aquatic tadpoles of *Hemisus* might be retention of the ancestral hemistoid + brevicipitid condition. The hemisotid condition also represents a plausible intermediate evolutionary step between the plesiomorphic afrobatrachian biphasic life history and the completely terrestrial development of brevicipitids. Several of the characters seen in *Hemisus*, such as jelly filled, egg-less egg capsules to prevent desiccation or physiological changes in nitrogen excretion of the tadpoles while in the terrestrial nest chamber (Grafe et al., 2005), might have served as a preadaptation to the fully terrestrial life history of brevicipitid frogs.

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