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Care and Parentage in a Skin-Feeding Caecilian Amphibian

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ABSTRACT An exceptional form of parental care has recently been discovered in a poorly known caecilian amphibian. Mothers of the Taita Hills (Kenya) endemic *Boulengerula taitanus* provide their own skin as a food source for their offspring. Field data suggest that nursing is costly. Females found attending young had a lower body condition and fat body volume than nonbrooding and egg-incubating females, and the female condition decreased substantially during parental care. Most mothers and their eggs or offspring were found in close proximity to other nesting females, in high-density nest sites that enhance the potential for social interactions and highlighting the possibility of communal breeding. Parentage was investigated using Amplified Fragment Length Polymorphism (AFLP) genetic markers in 29 offspring from six litters guarded by putative mothers. Our data provide the first evidence of multiple paternity in a caecilian, implying that two fathers sired one litter. Some young from two litters had genotypes not matching the guarding female suggesting that not all offspring are cared for by their biological mothers. This study provides evidence for alloparenting in an amphibian with cost-intensive parental care. *J. Exp. Zool. 309A:460–467, 2008.* © 2008 Wiley-Liss, Inc.

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Caecilians are superficially snake-like, primarily tropical amphibians that, because of their burrowing lifestyles, are mostly poorly known and are difficult to study (e.g. Himstedt, ’96; Gower and Wilkinson, 2005; Wake, 2006). Thus, only recently has it been discovered that some oviparous caecilians have extensive post-hatching parental care. In these species, initially pigmentless, altricial young tear off and eat the outermost layer of the hypertrophied skin of their attending mother within her nest chamber (Kupfer et al., 2006a; Wilkinson et al., 2008). Feeding of young (nursing) is commonplace in birds and mammals but relatively rare in other vertebrates (e.g. Clutton-Brock, ’91). Its discovery in caecilians calls for comparison with other taxa; however, very little is known yet about this intriguing amphibian system. Here we report new data on the breeding ecology, the energetic cost of nursing, and the first genetic evidence of multiple paternity and alloparenting (i.e. caring for the offspring of others).

MATERIAL AND METHODS

Study animal

Boulengerula taitanus is endemic to the Taita Hills of Kenya where it may be common in forest

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and agricultural soil ecosystems (Malonza and Measey, 2005). It is oviparous and iteroparous; eggs are laid in terrestrial nests and develop directly without an aquatic larval stage (Nussbaum and Hinkel, '94). As in other caecilians, fertilization is internal (e.g. Gower and Wilkinson, 2002; Wake, 2006) and females of oviparous species guard their eggs (e.g. Sarasin and Sarasin, 1887–1890; Kupfer et al., 2004). In *B. taitanus*, parental care is extended beyond hatching and includes nursing (Kupfer et al., 2006a). Initially altricial young bear a specialized, deciduous dentition used to feed on the stratum corneum of hypertrophied maternal skin (dermatophagy) during post-hatching parental care (Kupfer et al., 2006a). After dentitional metamorphosis, *B. taitanus* become generalist predators feeding on soil macrofauna such as termites, earthworms, and ants (Gaborieau and Measey, 2004). Although *B. taitanus* is the only caecilian for which an account of monthly collections over a full year has been published (Malonza and Measey, 2005), the duration of parental care, the extent of parental investment, and its energetic and other possible costs are not known.

Caecilian sampling and measurements

Collections of *B. taitanus* were made in the vicinity of Wundanyi (03°24.070′–03°24.935′S, 38°21.845′–38°22.971′E) in the Taita Hills of Southern Kenya at elevations of 1,270–1,450 m. Sampling was carried out after the short rainy season between 15–22 January 2004 and 10–18 January 2005 in small agricultural holdings (for details of other fieldwork conducted here, see Malonza and Measey, 2005). Each site was searched for caecilians by digging soil up to depths of 70 cm for 20–30 min with the assistance of local farmers. When *B. taitanus* were found within nest chambers, these were carefully excavated and the surrounding area then searched for others. If additional nest chambers were found within 50 cm, the area was considered a high-density nesting site. Nests were thus discovered and processed sequentially with care taken to avoid mixing of animals from different nests. Soil penetrability, pH, and temperature were measured at nest sites (see Kupfer et al., 2004) and the dimensions of nest chambers were measured to the nearest 5 mm. Soil texture was categorized following Dubbin (2001). In 2004, specimens were preserved upon collection, whereas in 2005 some were maintained and observed in captivity before preservation.

Sex was determined in anaesthetized (using MS222) adults by applying pressure posteriorly. Eversion of the phallus identifies males. The sex of some adults was later confirmed by dissection. The total length and mass of caecilians were measured to the nearest 1.0 mm and 0.1 g, respectively. To investigate the magnitude of the energetic cost of nursing in the field we calculated a body condition index (BCI) of adult females using the formula: $\mathrm{BCI} = 10^{-8} * (\text{body mass} * \text{total length}^{-3})$ (see Kupfer et al., 2004), which is based on the geometric scaling relation of *body mass–total length*^{3}. As a second proxy of energetic costs during parental care, we measured the total volume of the energy-storing (e.g. Exbrayat and Hraoui-Bloquet, 2006) fat bodies. We measured the dimensions (length and width) of fat body lobes in dissected specimens and estimated their volume using the formula $V = 4/3\ (L/2)(W/2)^2$. Each variable was tested for normal distribution using the D'Agostine and Pearson omnibus normality test. Paired and unpaired t-tests and one-way analysis of variance (ANOVA) were employed to compare means. To express a potential relation of normally distributed parameters, Pearson correlation coefficients were used and linear regression analysis was performed. All statistical tests were conducted using Minitab and Prism for Macintosh computers. Voucher specimens are deposited in the collections of The National Museums of Kenya, Nairobi (NMK A/4556/5 to A/4558/11).

Paternity analysis using AFLP markers

Given that co-dominant molecular markers (such as microsatellites) have not yet been developed for any caecilian, we applied dominant AFLP markers for genetic parentage analysis (generally following Whitlock et al., 2006). The dominant nature and low polymorphism of bi-allelic loci in AFLPs can be ameliorated by using a large number of loci (about one order of magnitude more than, for example, when using microsatellites) to provide estimates of parentage (Gerber et al., 2000; Bensch and Akesson, 2005).

DNA was extracted from skin using a standard phenol–chloroform procedure (Sambrook and Russell, 2001). Approximately 100 μg genomic DNA was digested with 1 unit each *EcoR*1 and *Taq*1, in a total reaction volume of 20 μL containing 2 μL 10 × TA buffer (100 mM Tris-Ac pH 7.9, 100 mM MgAc, 500 mM Kac, 10 mM DTT), and 5 μg bovine serum albumin, incubated at 37°C for 3 hr, followed by 65°C for 10 min. A 5 μL reaction mixture containing 0.5 units of T4 DNA ligase, 1 μL 5× ligation buffer, and 50 μM double-stranded adaptors of each *EcoR*1 (forward 5-CTCGTAGACTGCGTACC-3, reverse 5-AATT GGTACGCAGTCTAC-3) and *Taq*1 (forward 5-GA CGATGATCCTGAC, reverse 5-CGGTCAGGACT CAT-3) was immediately added to the digested DNA samples. Ligations of adaptors to restriction sites took place at 16°C overnight.

Pre-selective polymerase chain reaction (PCR) amplification was conducted in 10 μL reaction volumes, containing 2 μL of diluted (4:1) template DNA, 0.5 units *Taq* DNA polymerase (Thermo-primePlus, Advanced Biotechnologies, Columbia, MD) in the manufacturer's buffer (final concentrations 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween), 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, and 5 μM *EcoR*1 and *Taq*1 primers (EcoR1: GACTGCGTACCAATTC**T**, T1: GATGA GTCCTGACCGA**C**; the selective nucleotide is in bold). PCR amplification was performed in a Hybaid Touchdown thermal cycler (Thermo Hybaid, Ashford, Middlesex, UK), with a reaction profile of 120 sec at 94°C, followed by 20 cycles of 94°C (30 sec), 56°C (30 sec) and 72°C (60 sec), and a final 10 min at 72°C. The selective PCR was performed using 2.5 μL diluted (1:10) preselective PCR products, 2.5 mM $MgCl_2$, 0.5 units *Taq* DNA polymerase in the manufacturers' buffer (see above), and 5 uM selective *EcoR*1 (6-fam fluorescently labeled) and *Taq*1 primers carrying three selective nucleotides each (6-fam-GACTGCGTAC CAATTC**TCT**, in combination with GATGAGT CCTGACCGA**CTT**, GATGAGTCCTGACCGA**CTC**, GATGAGTCCTGACCGA**CAT**, and GATGAGT CCTGACCGA**CAG**, respectively). We used a touchdown thermal profile of 120 sec at 94°C, followed by 13 cycles of 94°C (30 sec), 65–56°C (30 sec, decreasing by 0.7°C per cycle), 72°C (60 sec) and 10 min incubation at 72°C. Fragments were separated and visualized using an ABI 3730 capillary sequencer and scored using the software GeneMapperTM Vision 3.5 (Foster City, CA) (ABI).

For each offspring, the putative origin (maternal or parental) of each polymorphic band in the DNA profile of a litter was determined by comparison with the maternal DNA fingerprint. For pairs of individuals, x and y, the similarity index S_{xy} was calculated as

$$S_{xy} = 2n_{xy}/(n_x + n_y)$$

where n_{xy} is the number of polymorphic bands shared by x and y, and (n_x+n_y) is the sum of the number of polymorphic bands in x and y (Lynch, '90). To avoid misclassification because of genotyping errors, pairs of individuals were only considered to be parent–offspring or full siblings if $S_{xy} \geq 0.4$, and less related if $S_{xy} < 0.4$ (Parrott et al., 2005). Paternity was determined by excluding all bands shared by mothers and young.

RESULTS

The majority of adult *B. taitanus* attending eggs/young were found in high-density nest sites: in 2004, 19 of 27 (70%) and in 2005, 29 of 34 (85%) adults attending clutches/litters were found in a site inhabited by groups of 1–12 other attending adults. Only 23% of attending adults were found with clutches/litters in more isolated nests. Nest densities ranged from 0.5 to 4 m^{-2}. Each nest contained a single adult with a litter of young or a clutch of eggs. Attending adults were invariably female, and a priori were assumed to be mothers of some or all of the attended eggs/young. Males and nonattending females comprised a small proportion (12 and 22%) of the adults found in high-density nesting sites.

Underground nests were ellipsoidal and, on average, 4 cm wide, 3.6 cm high, and 3.3 cm deep (mean = 0.211 l, SD = 0.094, $n = 12$), and were located at depths of 4–70 cm (median = 17 cm), typically in soil of a clay loam texture (pH 6.75), of low penetrability (0.20–1.20 kg/cm^2; mean = 0.73, SD = 0.30, $n = 29$), and temperatures of 17.6–21.1°C (mean = 19.39, SD = 0.75, $n = 30$).

In Year 1, 25 females were attending young (mean = 4 young, SD = 1, range = 1–6) and two were attending clutches of four eggs each. The total length of mothers was 214–307 mm (mean = 248.8 mm, SD = 20.6), and of young 35–94 mm (mean = 70.9 mm, SD = 14.8, $n = 88$, see Fig. 1). The young found later in January of Year 1 were larger than those found earlier in January of Year 2 (un-paired t-test, $t = 4.11$, $P < 0.001$). Only three young were found not in association with apparent parents, siblings, or nests. In contrast to all young found in nests, these were partly pigmented, relatively large (85–99 mm, see Fig. 1) and appeared independent (including an adult-type dentition and gut contents containing soil invertebrates), and are interpreted as likely having recently dispersed from their nests. In Year 2, 21 females were attending young (mean = 5 young per litter, SD = 2, range 2–9) and 13 were attending egg clutches (mean = 5 eggs, SD = 1, range 3–7). The total length of females ranged from 217 to 332 mm

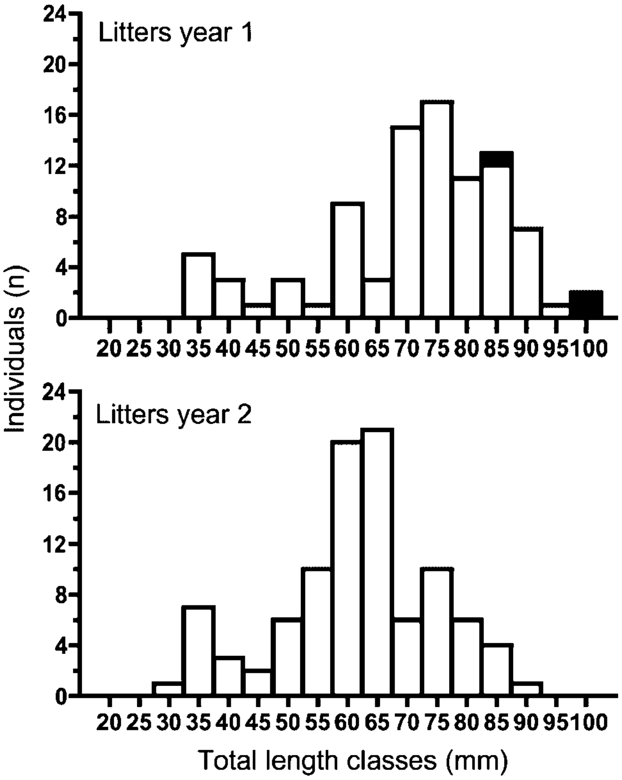

Fig. 1. Litter characteristics of *Boulengerula taitanus*. Frequency histograms of total length of offspring collected in January 2004 (25 litters, 88 young) and 2005 (21 litters, 97 young). White bars indicate all young found associated with their putative mother; black bars indicate solitary, unguarded young found in Year 1 ($n = 3$).

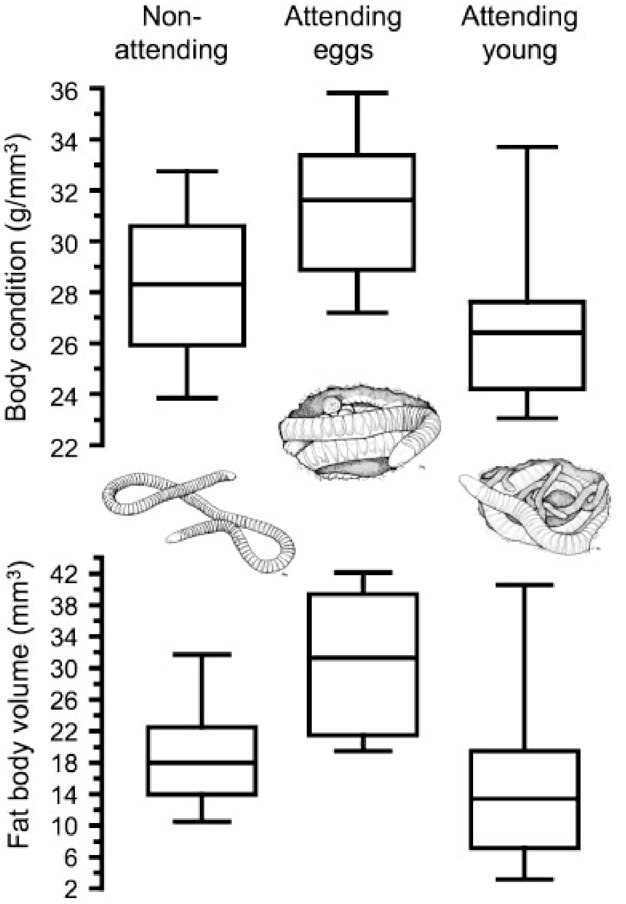

Fig. 2. Comparison of body condition and fat body volume among nonbreeding ($n = 15$), egg- ($n = 15$) and young-attending ($n = 21$) female *Boulengerula taitanus*.

(mean = 262.6, SD = 25.3), and of young found with putative mothers 30–88 mm (mean = 61.5, SD = 13.1, $n = 97$, Fig. 1). The uni-modal size distribution indicates a single cohort of young (Fig. 1). Between years there are no significant differences between numbers of eggs in each clutch or young in each litter, and there is no significant correlation between numbers of eggs/young and length of mother.

When collected, the body condition of mothers attending young was significantly lower than those of nonbreeding females and mothers attending egg clutches (one-way ANOVA: $F = 10.93$, df = 2, 45, $P < 0.001$, Fig. 2). Mothers attending egg clutches had a significantly higher body condition than nonattending females (un-paired t-test, $t = 2.86$, $P < 0.008$). The fat body volume of young attending females was significantly lower than those of nonbreeding females and mothers attending egg clutches (one-way ANOVA: $F = 16.75$, df = 2, 54, $P < 0.001$, Fig. 2).

The body condition of mothers attending young was negatively correlated with the mean total length of their offspring ($F = 18$, df = 1, 24, $p < 0.001$, $r^2 = 0.4390$, Fig. 3).

We analyzed parentage in six litters (A–F) collected from the largest high-density nest site in 2005, each containing from three to seven young (Table 1). We scored an AFLP size range of maximally 55–410 bp, with the number of polymorphic loci given in Table 1; because of some missing data (the selective amplification primer with the selective nucleotides CAG was not consistently readable in families A and F; see Material and Methods), we analyzed each family separately, rather than combining all loci across all families. Two offspring each from two litters (C and D) had a genetic similarity to the assumed mothers substantially less than expected (S_{xy} of < 0.4), suggesting that the females found with these young were not their genetic mothers (Table 1).

All 25 other young were likely the offspring of their attending females (Table 1). That adopted young fed on their attending females during post-hatching care observed in captivity (Kupfer et al., 2006a) was confirmed by dissection. Evidence for multiple paternity was present in one litter (all of which were the genetic offspring of the attending mother) in which two males likely sired two and five of the seven young (Table 2).

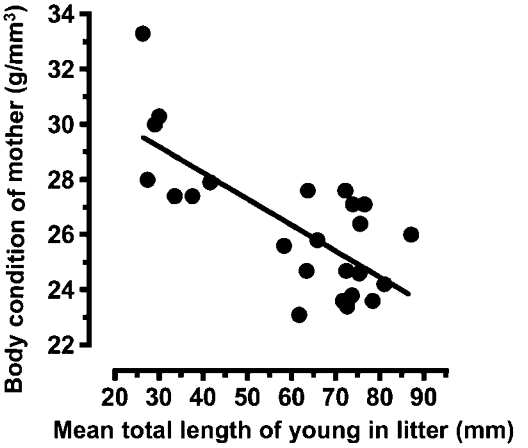

Fig. 3. Linear correlation between body condition of female *Boulengerula taitanus* and mean total length of their attended young ($n = 21$ litters) upon collection.

DISCUSSION

Kupfer et al. (2006a) used weight loss in captive brooding females of *B. taitanus* as evidence of the high cost of their parental care. That freshly caught animals have lower condition if they are breeding, and that condition decreases with the size of young (a proxy for the age of young and duration of care) demonstrates that maternal weight loss is not simply because of captivity. Young attending females also show a lower fat body volume (a proxy for energy storage). In contrast to Measey and Gower's (2005) study of the oviparous caecilian *Gegeneophis ramaswamii*, condition is a good predictor of the state of the fat bodies in *B. taitanus*.

TABLE 1. Relationships within six litters of Boulengerula taitanus revealed by AFLP analysis

Litter	*n* Polymorphic loci	*n* Young	Young 1	Young 2	Young 3	Young 4	Young 5	Young 6	Young 7	*n* Young directly related to mother (%)
A	140	4	0.55	0.44	0.41	0.42				4 (100)
B	154	7	0.94	0.54	0.61	0.74	0.69	0.77	0.49	7 (100)
C	211	5	0.29*	0.27*	0.85	0.88	0.88			3 (60)
D	223	5	0.85	0.83	0.82	0.24*	0.22*			3 (60)
E	93	5	0.92	0.96	0.95	0.81	0.75			5 (100)
F	23	3	0.61	0.56	0.58					3 (100)

Young (individuals numbered 1–7) sharing a similarity index (S_{xy} see text) of ≥ 0.4 were regarded as directly related to the mother they were found associated with in the nest sites. Those young having a similarity index $S_{xy} < 0.4$ were not considered as being produced by the putative mother (marked by *). The proportion of young directly related to the mother within a litter is given in the last column.

TABLE 2. Presence of multiple paternity in six litters of Boulengerula taitanus

Litter	*n* Young	Young 1	Young 2	Young 3	Young 4	Young 5	Young 6	Young 7	*n* Probable fathers
A	4	0.50/0.62/0.51	0.50/0.69/0.67	0.62/0.69/0.60	0.51/0.67/0.60				1
B	7	0.56/0.57/0.78/0.76/0.80/0.46	0.56/0.58/0.43/0.38/0.45/0.70	0.57/0.58/0.42/0.32/0.36/0.40	0.78/0.43/0.42/0.75/0.83/0.53	0.76/0.38/0.32/0.75/0.84/0.63	0.80/0.45/0.36/0.83/0.84/0.71	0.46/0.70/0.40/0.53/0.63/0.71	2
C	5	0.90/?/?/?	0.90/?/?/?	?/?/0.87/0.85	?/?/0.87/0.87	?/?/0.85/0.87			1
D	5	0.86/0.83/?/?	0.86/0.86/?/?	0.83/0.86/?/?	?/?/?/0.94	?/?/?/0.94			1
E	5	0.79/0.73/0.71/0.84	0.79/0.80/0.67/0.90	0.73/0.80/0.63/0.83	0.71/0.67/0.63/0.69	0.84/0.90/0.83/0.69			1
F	3	0.63/0.60	0.63/0.71	0.60/0.71					1

Similarity indexes between each young (numbered 1–7) and each of their litter mates are given (e.g. each young in a litter of four has a S_{xy} value relating to each of its three litter mates, denoted by *x*/*y*/*z*). Young that had a high proportion ($S_{xy} \geq 0.4$) of nonmaternally derived bands shared with litter mates are regarded to have the same father, whereas young with a low proportion of shared bands ($S_{xy} < 0.4$) are interpreted to have different fathers. Paternity analysis could not be run for young that were not directly related to the mother (denoted as "?", see Table 1). The number of probable fathers for each litter is shown in the last column.

These new results suggest that care is costly and that it depends upon stored reserves. Some of our observations extend those of Malonza and Measey (2005), most notably that animals as small as 214 mm may be reproductively active. We found no correlation between numbers of eggs and young and maternal length in the oviparous *B. taitanus*. On the other hand, clutch size is positively related to female length in the oviparous *Ichthyophis* cf. *kohtaoensis*. With viviparous species, Wake ('80) reported no relationship between the ovarian clutch size and the female total length in *Dermophis mexicanus*, whereas Moodie ('78) found the number of foetuses and maternal total length to be positively correlated in *Typhlonectes compressicaudus*. In summary, it is not clear to what degree the fecundity of caecilians is size related among different taxa and reproductive modes.

Our molecular genetic data permit the first analysis of parentage for a caecilian. Finding multiple paternity, and thus the potential basis for sperm competition, in *B. taitanus* is not surprising given that female investment is high and male investment apparently low, and that fertilization is internal. Multiple paternity is known in internal fertilizing salamanders such as *Plethodon cinereus* (Liebgold et al., 2006) and the viviparous *Salamandra salamandra* (Steinfartz et al., 2006).

Evidence of alloparenting is more unexpected. Only a few cases have been documented in amphibians, in egg-guarding salamanders such as *Hemidactylium scutatum* (Harris et al., '95; Harris and Ludwig, 2004) and *Ambystoma opacum* (Kaplan and Crump, '78; Croshaw and Scott, 2005). In these species, females oviposit eggs in communal nests and some females remain with the clutches until hatching, and there is no costly, post-paritive, maternal provision of nutrition. With *B. taitanus*, we suspect that alloparenting involves migration of young rather than multiple oviposition. With larger samples, this might be indirectly tested by comparing clutch sizes.

The significance of alloparenting is, at this stage, quite unclear but, because of nursing, its cost seems potentially much higher for *B. taitanus* than for salamanders. Broader surveys with co-dominant markers are needed to determine whether it is an occasional accident or whether it occurs frequently enough to be important in the reproductive ecology of the species. Alloparenting can be interpreted as misdirected parental care if parents cannot identify their own young, as in many fish (see Wisenden, '99). Kin recognition occurs in terrestrial salamanders exhibiting parental care (Masters and Forester, '95) and in the aquatic, viviparous caecilian *Typhlonectes natans* (Warbeck, 2002), which is not known to practice post-partum care. Kin recognition has yet to be studied in a terrestrial caecilian.

Alloparenting is common in animals that have a low reproductive output combined with cost-intensive parental investment (e.g. Helms Cahan et al., 2002). In birds and mammals (e.g. Riedman, '82) as well as in invertebrates (e.g. Eggert and Müller, '92; Scott, '98), alloparental care is well known from species living in small family groups and/or high-density breeding colonies. As far as is known, all oviparous caecilians lay eggs in subterranean nests and attend the eggs at least until hatching (Himstedt, '96). In *Ichthyophis* cf. *kohtaoensis* nests are mostly found in relative isolation (Kupfer et al., 2004), whereas nests of *B. taitanus* are more commonly found in close proximity to other nests. We do not know whether nest site quality is independent of the proximity of other nests, but proximity must increase the likelihood of interactions between individuals from different nests and could facilitate social interactions. The intriguing possibility that some form of cooperative breeding in these caecilians exists merits further investigation.

Although caecilians may be very challenging to study because of their burrowing life styles, obstacles are not insurmountable (e.g. Gower and Wilkinson, 2005; see also Kupfer et al., 2006b). Although preliminary, our data add to the sketchy knowledge of parentage patterns in other amphibians (e.g. Garner and Schmidt, 2003; Vieites et al., 2004). Our data suggest a system in which an energetically costly, novel form of post-hatching parental care is sometimes associated with high-density nesting sites and alloparental care. Further investigation of this unique amphibian system could provide a phylogenetically distant contrast to better-studied, analogous systems in birds and mammals (e.g. Helms Cahan et al., 2002; Komdeur and Heg, 2005), which could be used to test and extend our understanding of the evolution of parental care in vertebrates.

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