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Original Contribution

Batrachochytrium dendrobatidis Infection and Lethal Chytridiomycosis in Caecilian Amphibians (Gymnophiona)

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Abstract: *Batrachochytrium dendrobatidis* (*Bd*) is commonly termed the 'amphibian chytrid fungus' but thus far has been documented to be a pathogen of only batrachian amphibians (anurans and caudatans). It is not proven to infect the limbless, generally poorly known, and mostly soil-dwelling caecilians (Gymnophiona). We conducted the largest qPCR survey of *Bd* in caecilians to date, for more than 200 field-swabbed specimens from five countries in Africa and South America, representing nearly 20 species, 12 genera, and 8 families. Positive results were recovered for 58 specimens from Tanzania and Cameroon (4 families, 6 genera, 6+ species). Quantities of *Bd* were not exceptionally high, with genomic equivalent (GE) values of 0.052–17.339. In addition, we report the first evidence of lethal chytridiomycosis in caecilians. Mortality in captive (wild-caught, commercial pet trade) *Geotrypetes seraphini* was associated with GE scores similar to those we detected for field-swabbed, wild animals.

Keywords: Africa, Anura, Batrachia, Caudata, chytrid, pet trade, South America

Introduction

The approximately 7,000 extant amphibian species comprise three major clades, traditionally afforded the status of order in Linnean classification: the frogs and toads (Anura, ca. 6,250 extant species), newts and salamanders (Caudata, ca. 640 species), and caecilians (Gymnophiona, ca. 190 species) (e.g., www.amphibiaweb.org). The former two orders are sister taxa, together comprising Batrachia. Amphibian (or at least batrachian) populations worldwide are experiencing decline and extinctions, with approximately

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one-third of all species categorized as threatened on the IUCN Red List. The main recognized threats are habitat deterioration, climate change, human exploitation, invasive species, and disease. Among diseases reported to contribute to batrachian declines, the most prominent is the emerging infectious disease, amphibian chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). This near-globally ubiquitous (Farrer et al. 2011; www.bd-maps.net) skin parasite has been declared a major contributor to the global amphibian crisis (e.g., Berger et al. 1998; Skerratt et al. 2007; Lötters et al. 2010).

Although some authors have been careful to state that, for example, the "fungus infects 2 amphibian orders (Anura and Caudata)" (Hyatt et al. 2007: 175), *Bd* is commonly referred to as the 'amphibian chytrid fungus' even though it has been verified thus far infecting only anurans and caudatans (see Gower and Wilkinson 2005 for correction of one previous report in caecilians). Because Anura and Caudata are sister taxa, considering *Bd* the 'amphibian' (rather than batrachian) chytrid fungus is an extrapolation that remains largely untested. Although the assumption that *Bd* can infect all major lineages of amphibians has generally been made only implicitly, we assume it has been made because caecilians are amphibians, and thus expected to share features with the generally better-known batrachians.

Batrachochytrium dendrobatidis has been found to infect and develop within the epidermal cells of the stratum corneum and stratum granulosum of the skin of metamorphosed batrachian amphibians, and has been described predominantly as infecting ventral surfaces of terrestrial anurans and caudatans or the keratinized mouthparts of anuran larvae (e.g., Berger et al. 2004; Piotrowski et al. 2004). Although there are differences in detail (e.g., annular folds and the variable presence of dermal and subdermal scales: Taylor 1968) there is nothing about the general anatomy of caecilian skin (e.g., Fox 1983) that would suggest that *Bd* would be unlikely to infect these animals. The assumption that caecilians are epidemiologically equivalent to susceptible batrachians requires testing, given that resistance to infection has been documented within amphibian lineages known to be susceptible to *Bd* infection and chytridiomycosis (e.g., hylid anurans, Luquet et al. 2012).

Caecilians occur throughout the moist tropics except for Madagascar, Australasia and southeast Asia east of Wallace's Line (Taylor 1968). Caecilians are burrowers in soils as adults with the exception of four fully aquatic species of the South American Typhlonectidae. Assuming that all ichthyophiids and rhinatrematids have a biphasic life history, then approximately 35% of nominal species of caecilians are likely to have a more or less aquatic larval stage, with the remaining species being either direct developers or viviparous. Caecilians are not routinely encountered by 'standard' (e.g., Heyer et al. 1994) amphibian field surveys and dedicated effort is generally required to purposefully find them: for terrestrial caecilians this often involves digging soil (Gower and Wilkinson 2005). Unfortunately, published accounts of surveys for *Bd* in areas where caecilians occur do not allow for substantive statements regarding the susceptibility of this group. Caecilians have rarely been included in *Bd* field surveys and even then represented by very few specimens, none of which has tested positive (e.g., www.bd-maps.net; Doherty-Bone et al. 2008; Penner et al. 2013). The single exception to this is the study of Doherty-Bone et al. (2013) that reported the first molecular detections of *Bd* for field-swabbed caecilians, but these tests lack supporting histopathology and pathogen isolation, requirements for confirmation of infection with viable *Bd*.

Gymnophiona is the closest living lineage to Batrachia and determining whether or not caecilians also host *Bd* and have the potential to suffer from chytridiomycosis is required to determine if this parasite is a potential threat to all major extant amphibian lineages. Knowing this would represent a substantial advance in understanding the natural history of *Bd* and the conservation threat it poses. Approximately two thirds of caecilian species are categorized as Data Deficient on the IUCN Red List because of lack of information, including threats. Determining whether *Bd* is a threat could play an important part in making more informative conservation assessments for caecilians. Here we provide the first report of the molecular detection of *Bd* from wild-caught caecilians beyond Cameroon, including animals in the amphibian pet trade. We also present the first report of lethal chytridiomycosis in a caecilian, incorporating molecular detection, histological evidence, and culture of viable *Bd*.

Methods

In the period 2008–2011 we skin-swabbed more than 200 wild-caught caecilians (Table 1). A total of 198 animals were swabbed soon after capture in the field. Thirty-one wild-caught animals were swabbed after at least 2 years in captivity, but most of these were also swabbed when first

Table 1. Prevalence and GE of *Bd* in Sampled Caecilians.

Family	Genus	Species	Country	Locality	Altitude (m)	Year	Sample size	*Bd* +ve	Mean GE (total mean)
Typhlonectidae	*Typhlonectes*	*natans*	Colombia	Guarinócito	209	2008	8	0	
Caeciliidae	*Caecilia*	*tentaculata*	French Guiana	Kaw; Nouragues; Angoulême	50–300	2008	9	0	
Caeciliidae	*Caecilia*	*gracilis*	French Guiana	Nouragues	50–100	2008	1	0	
Caeciliidae	*Caecilia*	sp.	French Guiana	Nouragues	50–100	2008	1	0	
Rhinatrematidae	*Rhinatrema*	*bivittatum*	French Guiana	Kaw; Nouragues; Angoulême	50–300	2008	19	0	
Rhinatrematidae	*Rhinatrema*	*bivittatum*	French Guiana	Kaw; Nouragues	100–300	2011	3*	0	
Siphonopidae	*Microcaecilia*	*unicolor*	French Guiana	Kaw; Nouragues	100–300	2011	11*	0	
Siphonopidae	*Microcaecilia*	*unicolor*	French Guiana	Kaw	ca. 200	2008	1	0	
Siphonopidae	*Microcaecilia*	*unicolor*	French Guiana	Nouragues	50–100	2008	5	0	
Siphonopidae	*Microcaecilia*	*dermatophaga*	French Guiana	Angoulême	55	2008	8	0	
Siphonopidae	*Microcaecilia*	*dermatophaga*	French Guiana	Angoulême	55	2011	6*	0	
Rhinatrematidae	*Epicrionops*	sp.	Guyana	Iwokrama	ca. 800	2011	1	0	
Siphonopidae	*Microcaecilia*	sp.	Guyana	Iwokrama	50–900	2011	8	0	
Siphonopidae	*Caecilita*	*iwokramae*	Guyana	Iwokrama	50–900	2011	13	0	
Dermophiidae	*Geotrypetes*	*seraphini*	Cameroon	Doumo-Pierre	641	2009	1	0	
Dermophiidae	*Geotrypetes*	*seraphini*	Cameroon	Kon	ca. 600	2011	7**	0	
Dermophiidae	*Geotrypetes*	*seraphini*	Cameroon	Ndikinimeki	ca. 800	2008	14	9†	0.242–14.104 (3.123)
Dermophiidae	*Geotrypetes*	*seraphini*	Cameroon	Ntengue	ca. 800	2008	5	4†	2.42–14.338 (10.593)
Herpelidae	*Herpele*	*squalostoma*	Cameroon	Dja	ca. 650	2009	2	0	
Herpelidae	*Herpele*	*squalostoma*	Cameroon	Kon	ca. 600	2011	5**	0	
Herpelidae	*Herpele*	*squalostoma*	Cameroon	Ndikinimeki	ca. 800	2008	2	1†	17.339
Herpelidae	*Herpele*	*squalostoma*	Cameroon	Mundame	ca. 50	2008	19	10†	1.77–4.87 (2.672)
Herpelidae	*Herpele*	*squalostoma*	Cameroon	Banga Bakundu	56	2008	7	7†	0.277–3.79 (1.512)
Indotyphlidae	*Idiocranium*	cf. *russeli*	Cameroon	Ndikinimeki	ca. 800	2008	27	18†	0.054–13.383 (2.454)
Indotyphlidae	*Idiocranium*	cf. *russeli*	Cameroon	Ndikinimeki	ca. 800	2011	11*	0	
Scolecomorphidae	*Crotaphattrema*	*lamottei*	Cameroon	Mt. Oku	ca. 2,150	2008	6	2†	0.071–6.215 (3.143)
Dermophiidae	*Schistometopum*	*gregorii*	Tanzania	Bagamoyo	<50	2008	7	0	
Herpelidae	*Boulengerula*	cf. *uluguruensis*	Tanzania	Nguru (Pemba)	950–1,050	2008	7	4†	0.59–1.19 (0.896)
Herpelidae	*Boulengerula*	cf. *uluguruensis*	Tanzania	Nguu (Nguu North FR)	ca. 1,350	2008	13	1†	0.144

Table 1. continued

Family	Genus	Species	Country	Locality	Altitude (m)	Year	Sample size	*Bd* +ve	Mean GE (total mean)
Herpelidae	*Boulengerula*	cf. *uluguruensis*	Tanzania	Uluguru (Uluguru North FR)	995	2008	5	0	
Herpelidae	*Boulengerula*	sp.	Tanzania	Pet Trade	?	2009	3	0	
Scolecomorphidae	*Scolecomorphus*	cf. *kirkii*	Tanzania	Nguru (Maskati)	ca. 1,500	2008	5	1†	0.648
Scolecomorphidae	*Scolecomorphus*	cf. *kirkii*	Tanzania	Nguu (Nguu North FR)	ca. 1,350	2008	4	1†	0.052
Scolecomorphidae	*Scolecomorphus*	cf. *kirkii*	Tanzania	Uluguru (Uluguru North FR)	1,200	2008	3	0	

Mean GE values given only for *Bd*-positive samples. All animals are terrestrial except for the *Typhlonectes natans* and all post-metamorphic except for 8 of the *Rhinatrema bivittatum* swabbed soon after capture.
FR Forest Reserve.
⋆Swabbed in captivity but mostly/all also swabbed when caught in wild.
⋆⋆Wild-caught but not swabbed until after a substantial period of captivity.
†All were negative in the absence of BSA additive in PCR reactions.
See "Appendix" for locality coordinates.

captured in the field. Twelve wild-caught animals from Kon, Cameroon were not swabbed upon capture, but were swabbed only after nearly 3 years in captivity, and three (presumably wild-caught) *Boulengerula* sp. that were obtained through the pet trade were also swabbed only in captivity. In captivity, multiple individuals were kept in single-species communities in moist, sterilized topsoil, fed with live invertebrates, and maintained on a 12–12 h inverse light cycle at approximately 25°C (within the range of 22–28°C).

Swabbed individuals include representatives of 8 of the 10 (Wilkinson et al. 2011; Kamei et al. 2012) caecilian families, 12 of the 34 genera and approximately 10% of the 190 nominal species (Table 1). Geographic coverage was very patchy, comprising only Africa (Tanzania, Cameroon) and northern South America (Colombia, Guyana, French Guiana). Sample sizes were generally small for any species at a given locality, ranging from 1 to 27 (mean 7.6). With the exception of eight larval *Rhinatrema bivittatum*, all of the swabbed caecilians were post-metamorphic, and most of the swabbed species lack a larval stage. Many specimens were collected in very wet mud or seepages, but the only fully aquatic animals swabbed were eight adult *Typhlonectes natans*. All wild-caught terrestrial animals were found by digging in soil, except for approximately 10 animals from French Guiana that were encountered moving on the surface, mostly on roads at night and during or soon after heavy rain.

Because sampling for *Bd* was ancillary to the primary goals of the field studies, sterile technique was not strictly observed. Violations included sometimes keeping multiple animals of the same species in the same plastic bag for short periods (generally less than one day, in local soil), and generally not handling animals with sterile gloves at time of or after capture. All of the wild-caught, field-swabbed animals were initially handled using bare hands. The vast majority of caecilians were handled separately from anurans during capturing and swabbing procedures, no caecilians were stored in bags with frogs at any time, and at most localities (Colombia; French Guiana; Guyana; Bagamoyo and Maskati in Tanzania; Oku, Ntengue and Banga Bakundu in Cameroon) anurans were not part of the research exercise and can be excluded as potential sources of DNA contamination. A more stringent sterile technique was employed in the Cameroon fieldwork (Doherty-Bone et al. 2013). The eight *T. natans* and three pet-trade *Boulengerula* sp. were swabbed with cosmetic cotton buds, all other animals were swabbed with fine tip, sterile

rayon-tipped swabs (MW100-100; Medical Wire & Equipment Co, Crosham, UK).

Swabbing was done by two people. One person held the caecilian by the head and posterior end and tried to stretch it out. The other person swabbed along the length of the body, contacting ventral, lateral, and dorsal surfaces with the swab. Many caecilians (approximately half) were anesthetized by immersion in an aqueous solution of MS222 (Sandoz) before swabbing. Excess anesthetic solution was shaken and/or wiped off (sometimes with sterile gloves) but the same solution was generally used for multiple animals from the same locality. Soil was removed from caecilians prior to swabbing by rinsing in tap water and/or cleaned with moist sterile gloves or bare hands. Swabs were stored dry, dark and away from heat, the tips of the 11 cotton buds (animals from Colombia and the pet trade) were stored in vials of 95% ethanol.

In the laboratory, DNA was extracted from swabs following the protocol given by Boyle et al. (2004). Samples were subjected to quantitative real time polymerase chain reaction (qPCR) diagnostic assay, using *Bd* primers specific to the ITS-1/5.8S region of ribosomal gene (Boyle et al. 2004). Positive controls of known concentration of *Bd* DNA (100, 10, 1, and 0.1 *Bd* zoospore genomic equivalents—GE) were run as standards along with the samples, as were negative controls. Samples were run in duplicate on PCR plates and, if necessary, were repeated until both wells for each sample gave the same (positive or negative) result. Bovine serum albumin (BSA) was included in PCR reactions to reduce amplification inhibition (Garland et al. 2010) for all DNA extracts, but a subset (see Table 1) were run initially without BSA. Assay results are deposited in the *Bd* Global Mapping Project (www.bd-maps.net).

On 13 November, 2012, we acquired 19 pet trade *Geotrypetes seraphini* that had been recently imported from Cameroon directly to the UK by a licensed importer. These animals had been housed for slightly less than two weeks by the importer before we acquired them, and they were not cohoused with any other amphibians during shipping to or while maintained in the UK All 19 *G. seraphini* were swabbed the day after arrival at the Institute of Zoology, ZSL, following the procedure outlined above. The 18 animals surviving three weeks later (7 December) were reswabbed, and two of the animals that we suspected were infected were reswabbed using an alternative protocol. For these two animals, we used separate swabs to sample the head (all surfaces), anal disc, dorsal surface of the body, and ventral surface of the body. All swabs were subject to the same extraction and PCR protocol outlined above. In early December, several animals began to exhibit signs of ill health. Animals entered into veterinary care, but three animals died before antifungal treatments were started, and one died early on during treatment. All dead animals were subject to full gross *post mortem* examination plus parasitological and bacteriological testing and histological examination of skin. Histological examination of internal organs was carried out for two specimens. Dead specimens were deposited in the Zoological Society of London Pathology Archive (accessions ZA/1100/12, ZA/1101/12, ZA/1102/12, ZA/1107/12). In addition, skin samples taken from the head, anal disc, and dorsal body surface of all three of these animals we used in an attempt to isolate and culture *Bd*, following the protocols used by Farrer et al. (2011). In brief, larger skin samples were cut into pieces 1–2 mm in length and width and cleaned by dragging them through TGhL (tryptone, gelatin hydrolysate, and lactose) agar-containing antibiotics (penicillin-G and streptomycin sulfate) several times. Cleaned pieces were then transferred to 12-well plates containing liquid TGhL with antibiotics, and wells were checked every second day for zoospore activity or until bacterial or other fungal growth had occurred.

Results

Batrachochytrium dendrobatidis was detected on swabs of 58 individuals, approximately 30% of the sample, not including the 19 *G. seraphini* acquired in 2012 (Table 1). *Batrachochytrium dendrobatidis* was not detected on any captive caecilian sampled before 2012, irrespective of whether these animals were part of a sample for which *Bd* was detected from swabs taken soon after capture in the field. *Batrachochytrium dendrobatidis* was detected on caecilians sampled in the two African countries, but not from animals sampled in South America. We detected *Bd* on all African species except Tanzanian *Schistometopum gregorii* and the populations of *Boulengerula* and *Scolecomorphus* from the Uluguru mountains. The Tanzanian positives include the first reported for any amphibians from the Nguru and Nguu mountains.

Infectious burdens of field-sampled animals were relatively low (GE 0.052–17.339), but were, on average, an order of magnitude greater for Cameroon (0.054–17.339, mean 3.443) than for Tanzania (0.052–1.19, mean 0.35). The prevalence of *Bd* was also much higher for Cameroon

Table 2. Prevalence and GE of *Bd* in African Caecilians Swabbed Soon After Capture (i.e., Excluding Caecilians Held in Captivity), Grouped Taxonomically.

Country	Genus	Sample	*Bd* positive	Prevalence%	GE (mean)
Tanzania	*Boulengerula*	25	5	20	0.59–1.19 (0.745)
Tanzania	*Schistometopum*	7	0	0	
Tanzania	*Scolecomorphus*	12	2	16.7	0.052–0.648 (0.35)
Tanzania	All	44	7	15.9	0.052–1.19 (0.633)
Cameroon	*Crotaphatrema*	6	2	33.3	0.072–6.215 (3.143)
Cameroon	*Geotrypetes*	20	13	65	0.639–14.338 (5.421)
Cameroon	*Herpele*	30	18	60	0.277–17.339 (3.036)
Cameroon	*Idiocranium*	27	18	66.7	0.054–13.383 (2.454)
Cameroon	All	83	51	61.4	0.054–17.339 (3.443)

GE values given only for positives.

(64%) than for Tanzania (16%) (Table 2). Frogs were also swabbed during some of the same field expeditions in Africa that caecilians were sampled. For Cameroon many *Bd* positives were recorded for frogs (see Doherty-Bone et al. 2013) but none for Tanzania (unpublished data). Informative comparisons are limited because qPCR for the Tanzanian frog swabs were not conducted using BSA. Although the prevalence of *Bd* in caecilians was substantially higher than for frogs in the Cameroon sample, there was only very patchy overlap in localities sampled and Doherty-Bone et al. (2013) found no significant differences in the prevalence or GE of *Bd* between sympatric frogs and caecilians. Courtois et al. (2012) have reported low prevalence of *Bd* in frogs in two of the localities in French Guiana (Kaw, Nouragues) from which we sampled caecilians (all negative for *Bd*) in this study.

Three of the 19 pet trade *G. seraphini* sampled in 2012 tested positive for infection when first swab sampled on 14 November (GE scores 0.30 ± 0.15, 0.62 ± 0.11, and 7.10 ± 1.35) and the animal with the highest GE score died before the 7 December reswabbing. One of two duplicate amplifications was successful for another four animals for the 14 November swabs, but we could not establish infection status of these animals unambiguously despite repeated attempts to amplify using the same dilution and additional dilutions of the stock extractions. Reswabbing three weeks later resulted in unambiguous positive tests for two of these four animals (GE scores 3.92 ± 0.55, 111.74 ± 1.45), plus an additional two animals that had initially (14 November) tested as clear negatives (GE scores 0.22 ± 0.16, 78.33 ± 16.8). Only one of the two surviving animals that initially (14 November) tested positive still tested positive three weeks later, and for this animal infection was detected on the head (GE 0.69 ± 0.03), anal disc (GE 0.21 ± 0.10), and dorsal body surface (GE 0.36 ± 0.02), but not on the ventral surface of the body. The animal that tested positive on 14 November but negative on 7 December died soon afterwards. The animal that died during antifungal treatments was the animal with the highest GE score overall (second swab, GE 111.74 ± 1.45).

One of the *G. seraphini* subjected to *post mortem* examination exhibited signs consistent with chytridiomycosis (skin erosion on the dorsal surface of the head), and histological evidence of *Bd* infection was found in this animal and two others that died before treatment. H & E- and PAS-stained sections (5 μm thick) revealed multifocally extensive, moderate to marked disorganization and hyperplasia and dysplasia of the epidermis, hyperkeratosis, individual and clustered oval, flask-shaped or crescentiform sporangia, approximately 10–25 μm in diameter, located within the hyperplastic epidermis or keratin. Some sporangia were filled with endospores approximately 2–5 μm in diameter (Fig. 1), others were empty and some appeared triseptate and in some cases we observed what appeared to be sporangia discharge tubules (Fig. 2). Skin from the head, anal disc, and dorsal body surface all exhibited some or all of these signs. Additional indications of infectious diseases included infection of the lung lumen with an unidentified nematode in one animal and rhinitis in the nasal passage of another. Viable zoosporic fungus was successfully isolated from skin taken from the head of one of the animals that died before the onset of antifungal treatment. Initial indications of growth were zoospore activity three days after skin sections were placed in liquid culture. During

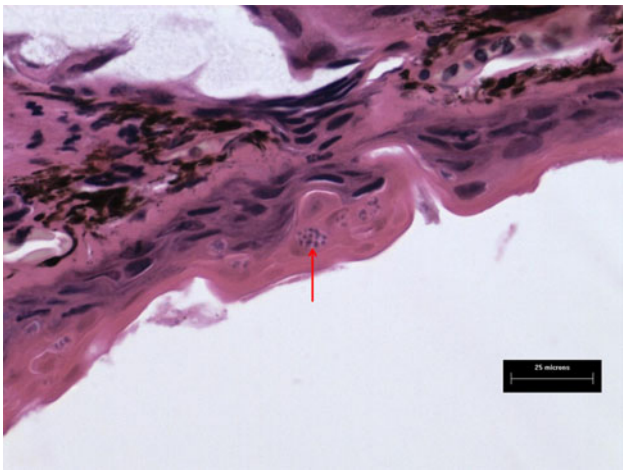

Figure 1. H & E-stained section of head skin of *Geotrypetes seraphini* that died before the start of antifungal treatments. *Arrow* points to oval, spore-filled sporangium within an area of skin exhibiting hyperkeratosis.

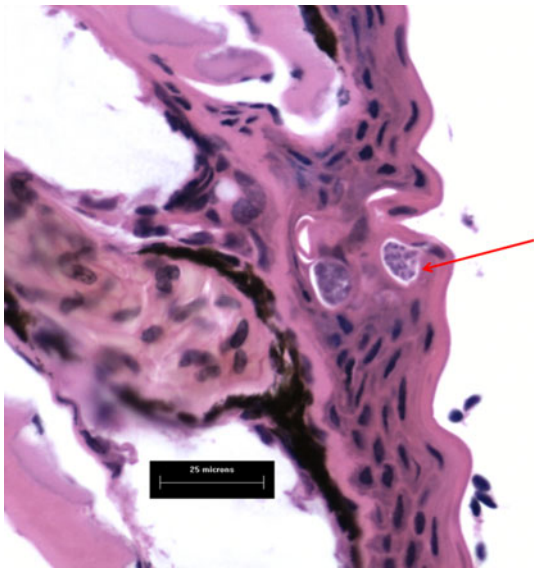

Figure 2. H & E-stained section of skin from the anal disc of *Geotrypetes seraphini* that died before the start of antifungal treatments. *Arrow* points to oval, spore-filled sporangium with discharge tubule within an area of skin exhibiting epidermal hyperplasia.

subsequent subculturing we observed various stages of sporangial development consistent with previous observations of *Bd* cultured from batrachians (Fisher et al. 2009b; Farrer et al. 2011), including immature stages, pseudohyphae, and mature sporangia containing active zoospores. We estimate it took five to six days to complete the life cycle from zoospore to zoospore.

Discussion

Our results show that *Bd* infection occurs in wild and pet trade African caecilians. Without histological or immunohistochemical examination we cannot unambiguously confirm that the *Bd* detected in field specimens was not contamination. The general lack of gloves and relaxed sterile technique in this study are not ideal sampling protocol, but the few studies that have been conducted thus far have indicated that contamination from washed bare hands is unlikely and that most false positives are the result of contamination during laboratory procedure (e.g., Skerratt et al. 2011), for which our negative controls present no evidence. Unless they are very abundant and/or there are a large number of people involved, then handling terrestrial caecilians with sterile gloves at all stages of field collection is unrealistic. Thus, refined assessments of the impact of environmental *Bd* on qPCR assays might be important for future research on infection of caecilians by *Bd*. However, the GE scores derived from field-swabbed animals, including *G. seraphini*, are broadly consistent with those we derived from the pet trade *G. seraphini* sampled in 2012. Histopathology confirmed the presence of infection with fungal sporangia containing zoospores in the 2012 *G. seraphini* and signs of skin disease were consistent with chytridiomycosis observed in anuran amphibians. Furthermore, we successfully isolated viable, zoosporic fungus from one of these 2012 *G. seraphini*, and the life-history stages and length of time required to complete the life cycle we observed for the cultured fungus all are consistent with what has been observed (Fisher et al. 2009b; Farrer et al. 2011) in isolating and culturing *Bd* from batrachian amphibians. The confirmation of qPCR results in the 2012 sample and the similarity in GE scores between the 2012 captive animals and those we sampled in the field, including conspecifics in both data sets, leads us to conclude that, *ceteris paribus*, field detections indicate infection in the wild. The seven positive *Bd* results for Tanzanian caecilians were initially negative when BSA was not included in the assay. This highlights the potential benefits of increased assay sensitivity with this additive, and the 100% negative results for the negative controls does not point to an increased problem of false positives.

Along with Doherty-Bone et al. (2013), this is the first report of *Bd* in field-swabbed and terrestrial caecilians. Although *Bd* and chytridiomycosis has been reported in some predominantly or strictly terrestrial anuran and caudatan amphibians (e.g., García et al. 2009; Vazquez et al.

2009; Weinstein 2009; Longo and Burrowes 2010), *Bd* is considered to be an aquatic pathogen (e.g., Gower et al. 2012). Our positive *Bd* results recorded for soil-dwelling caecilian species that lack aquatic life-history stages emphasize the more ecologically widespread nature of *Bd*. Raphael and Pramuk (2007) used qPCR to diagnose infection with *Bd* in 13 of 24 swabbed captive, aquatic *Typhlonectes natans* (a confiscated shipment from Colombia: J. Pramuk, pers. comm., 2012). Although *Bd* was detected from swabs it is unknown whether this was superficial and/or environmental contamination or whether these *T. natans* were infected. Raphael and Pramuk (2007) also found that none of these *T. natans* was positive for *Bd* after 72 h and after the environmental temperature was increased from 21 to 24.5°C to 32.2°C. The absence of *Bd* in our sample of captive caecilians collected from *Bd*-positive field localities before 2012 could be explained by *Bd* dying or surviving at undetectable levels in captive conditions. This seems unlikely, at least for the *G. seraphini* in captivity before 2012, given the rapid progression of infection and disease in the 2012 captives. The alternative explanation that all of the captive animals were *Bd*-negative when captured in the field is also unlikely because, for example, *Bd* prevalence among *Idiocranium* cf. *russeli* was approximately 70% for freshly captured animals (sample size, $n = 27$) but 0% ($n = 11$) for captive animals. Some, perhaps all, of the latter were among the 27 individuals of *I.* cf. *russeli* that were swabbed upon capture, and so likely included *Bd*-positive specimens entering captivity. Whatever may be influencing overall prevalence in captive caecilians, the fact remains that in at least one case, animals that had entered into the commercial pet trade tested positive for infection with an apparently virulent form of the fungus. Caecilians are nowhere near as prevalent in the amphibian trade as batrachians, but interest in them goes beyond the most commonly encountered species, *T. natans* (e.g., Gower and Wilkinson 2005).

The discovery of amphibian chytridiomycosis and then *Bd* was motivated by finding dead frogs in the field (e.g., Berger et al. 1998; Lips 1999; Longcore et al. 1999). There are no reports of similar phenomena in caecilians, but they would be less likely to be noticed given the more cryptic habits, low encounter rates and relative lack of research on these amphibians. Those of us who have carried out substantial caecilian fieldwork (DJG, TD-B, SPL, MW, MTK, HM) have together found a total of not more than five obviously diseased or dead (but not killed accidentally or purposefully by humans) caecilians during thousands of person hours of dedicated caecilian fieldwork in approximately 20 countries over the past 17 years, and know of no other finds by other researchers. Our observations of mortality in the captive *G. seraphini* in 2012, though, raises the strong possibility that the absence of field observations of lethal chytridiomycosis is not representative of the potential for this disease to be a threat to caecilians. Four out of 18 captive animals died and the only signs of disease shared among the three animals examined *post mortem* were consistent with chytridiomycosis. All of the 2012 *G. seraphini* were housed individually once they arrived at the Institute of Zoology and were being managed following strict biosecurity, so transmission among these animals after we began sampling was very unlikely to have contributed to the progression of infection we observed. Instead, infections developed without the benefit of forcing through among-host transmission and, in some cases, detectable infections arose from previously undetectable infections already carried by some animals. Thus we cannot disregard the possibility that all the 2012 pet trade *G. seraphini* were infected with *Bd* and, by extension, that field estimates of prevalence based on standard skin swabbing methods are an underestimate of the true prevalence.

Reviews of *Bd* biology that have outlined future research needs (e.g., Kilpatrick et al. 2010) have not highlighted addressing the lack of information of *Bd* in caecilians as a priority, but we suggest it should be one. Among extant amphibians, caecilians are ecologically and morphologically disparate to an extent that needs to be taken into consideration when planning future research. Questions to be addressed include: which caecilians succumb to amphibian chytridiomycosis and is it a conservation threat; what are the criteria for lethal sampling for histology; and what is an appropriately sterile swabbing technique in the field? Results of reswabbing captive *G. seraphini* in 2012 highlight that where, how, and how often a caecilian is swabbed also needs to be carefully considered. Both qPCR and histology indicate that the ventral surface of the caecilian body is a poor target for detection even though, along with limbs and digits that caecilians lack, it has been a recommended area to swab sample in batrachian amphibians (e.g., Smith 2011). While the generality of our finding for captive *G. seraphini* requires confirmation, our results suggest that the head, dorsal body surface, and anal disc may be more appropriate for swab sampling of caecilians. In the longer term, worthwhile studies of caecilians would include variations in infection by (and response to) *Bd* with respect to broad ecological types (e.g., aquatic vs.

terrestrial), reproductive and life-history modes (e.g., biphasic vs. direct development), surface area to volume ratios, skin peptides (not yet explored in caecilians), and degree of dermal and subdermal scalation. This is in addition to studies that are also of relevance to those being carried out for other amphibians, including variation in chytridiomycosis with environmental change, including seasonality (Berger et al 2004; Kriger and Hero 2007; Conradie et al. 2011). The disparity of caecilians and batrachians might make the former especially useful in gaining new comparative insights into *Bd* biology. Studies of caecilians might, for example, help to resolve uncertainty (e.g., Fisher et al. 2009a; James et al. 2009; Farrer et al. 2011) in the origins, evolution and pathogenicity of *Bd*.

To conclude, we recommend that: (1) more caecilians (individuals and taxa) are subjected to *Bd* diagnostic surveys; (2) experimental trials are conducted in which *Bd*-negative caecilians are challenged with *Bd* zoospores in order to establish possible outcomes of exposure (e.g., infection, disease); and (3) isolates of *Bd* should continue to be recovered from natural caecilian populations to ascertain the relatedness of the strain(s) infecting this amphibian group to global patterns of genetic variation seen in *Bd* infecting batrachians.

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Appendix

Coordinates in degrees for localities are included in Table 1. Data for each locality are approximate and given to two decimal places.

Cameroon

Banga Bakundu (4.41 N, 9.45 E), Dja (3.39 N, 13.12 E), Doumo-Pierre (3.47 N, 13.06 E), Kon (4.83 N, 11.06 E), Mt. Oku (6.22 N, 10.46 E), Mundame (4.57 N, 9.51 E), Ndikinimeki (4.75 N, 10.82 E), Ntengue (5.37 N, 10.02 E).

Colombia

Guarinócito (5.34 N, 74.74 W).

French Guiana

Angoulême (5.41 N, 53.65 W), Nouragues (4.06 N, 52.68 W), Kaw (4.54 N, 52.18 W).

Guyana

Iwokrama (4.33 N, 58.8 W).

Tanzania

Bagamoyo (6.48 S, 38.82 E), Maskati (6.06 S, 37.48 E), Nguu North FR (5.49 S, 37.49 E), Pemba (6 S, 37.55 E), Uluguru North FR (6.94 S, 37.71 E).

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