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***Caecilita* Wake & Donnelly, 2010 (Amphibia: Gymnophiona) is not lungless: implications for taxonomy and for understanding the evolution of lunglessness**

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Abstract

According to current understanding, five lineages of amphibians, but no other tetrapods, are secondarily lungless and are believed to rely exclusively on cutaneous gas exchange. One explanation of the evolutionary loss of lungs interprets lunglessness as an adaptation to reduce buoyancy in fast-flowing aquatic environments, reasoning that excessive buoyancy in such an environment would cause organisms being swept away. While not uncontroversial, this hypothesis provides a plausible potential explanation of the evolution of lunglessness in four of the five lungless amphibian lineages. The exception is the most recently reported lungless lineage, the newly described Guyanan caecilian genus and species *Caecilita iwokramae* Wake & Donnelly, 2010, which is inconsistent with the reduced disadvantageous buoyancy hypothesis by virtue of it seemingly being terrestrial and having a terrestrial ancestry. Re-examination of the previously only known specimen of *C. iwokramae* and of recently collected additional material reveal that this species possesses a reasonably well-developed right lung and is a species of the pre-existing caecilian genus *Microcaecilia* Taylor, 1968. We therefore place *Caecilita* in the synonymy of *Microcaecilia*, and re-evaluate the plausibility of the reduced disadvantageous buoyancy hypothesis as a general explanation of the evolution of lunglessness.

Key words: amphibian, *Atretochoana*, buoyancy, Guyana, lunglessness

Introduction

Lungs are an ancestral feature of Tetrapoda and are almost ubiquitous among living tetrapods. Secondary lunglessness is a rare condition that is currently considered to have evolved independently five times within amphibians, twice in caecilians, twice in salamanders and once in frogs (Hutchison, 2008; Wake & Donnelly, 2010), but nowhere else within the tetrapods. Explanations of the evolution of this unusual condition have mostly suggested that lunglessness is or was an adaptation to life in fast-flowing waters where pulmonary buoyancy, and concomitant difficulty in maintaining position, could be positively disadvantageous (e.g., Wilder & Dunn, 1920; Wilkinson & Nussbaum, 1997; Bickford *et al.*, 2008). This is a plausible, if controversial, explanation of lunglessness in amphibians (Hutchison, 2008) with the clear exception of lunglessness in the recently discovered monotypic caecilian genus *Caecilita*. *Caecilita iwokramae* Wake & Donnelly, 2010 appears to be a small terrestrial species belonging to a family of entirely terrestrial, directly developing caecilians (Wilkinson *et al.*, 2011). As such, its reported lunglessness cannot be readily explained by reference to an aquatic lifestyle and *Caecilita iwokramae* therefore represents the greatest empirical challenge to the generality of the reduced disadvantageous buoyancy (RDB) hypothesis for the origin of lunglessness in amphibians.

Caecilita iwokramae was described on the basis of a single specimen from the Iwokrama Rainforest in Guyana. It was reported to lack lungs, pulmonary arteries and veins, and have sealed internal and external nostrils. Sealed external nostrils have not been found in any other tetrapod and, together with the other unusual features, were used to justify recognition of the new monotypic genus *Caecilita*. However, our re-examination of the type

specimen of *C. iwokrama*e and of new material collected from the type locality reveal that important parts of the original description are inaccurate. Here we report our observations on the anatomy of *C. iwokrama*e, revise its taxonomy, and consider the significance of our findings for understanding of the evolution of lunglessness.

Materials and methods

The holotype of *Caecilita iwokrama*e was examined, and photomicrographs prepared, using a Zeiss Discovery V12 microscope. Measurements were made using dial calipers and/or with Axiovision software. Fieldwork at the type locality was conducted between the 17/03 and 22/03/2011, with caecilians sought by digging with hoes. Specimens were euthanized by immersion in a solution of MS222, fixed in 10% formalin for a few days, rinsed in water and transferred to 70% ethanol for permanent storage. Skulls of the holotype and newly collected material and aspects of soft anatomy (the latter after brief submersion in Lugol's solution to temporarily enhance contrast between the skin and the external medium) were visualized using non-destructive, high-resolution x-ray computed tomography (HRXCT) using a Metris X-Tek HMX ST 225 System at the Natural History Museum, London and post-processing with the Drishti software (Limaye, 2006). Observations of the viscera of the holotype were made through pre-existing incisions, reflecting organs where necessary. Additional dissection of the holotype was limited only to teasing apart connective tissue to reveal the course of pulmonary blood vessels. A drop of methylene blue was applied to the right external naris of the holotype to help temporarily visualize this structure and its connections under light microscopy.

Results

Morphology of the holotype of *Caecilita iwokrama*e. As originally reported, the holotype is a small adult specimen (c. 111 mm total length). It is somewhat faded and partially dissected with two longitudinal ventral incisions into the coelom and some deep dorsal transverse cuts posteriorly. The original description is accurate in most respects, but our observations on the respiratory and cardiovascular systems differ crucially and substantially from the original description of the holotype. We found that the external nares, although small and difficult to discern, are open (patent) and not sealed as claimed. Observations to this end made with unenhanced light microscopy were confirmed by HRXCT (Fig. 1a) and by the take-up of methylene blue through the nostril under light microscopy (Fig. 1b). The choanae (internal nostrils) are also difficult to see, but, also in contradiction of the original description, they are not sealed. Rather, they are equipped with small valves (not shown) as in all other terrestrial caecilians.

The holotype of *Caecilita iwokrama*e possesses a single, reasonably well-developed lung situated in the expected position just caudal to the heart and dorsal to the anteriormost part of the liver (Fig. 1c). Several colleagues (including Ralf Britz, Emma Sherratt, and Gabriela Bittencourt) independently examined and confirmed the presence of a lung. The lung of the holotype of *C. iwokrama*e has the characteristic morphology of a caecilian lung, being much longer (c. 9.5 mm) than wide (c. 0.4 mm) and including intrinsic cartilages that are present throughout most of its length. The last few millimetres of the lung are perhaps unusual in lacking these cartilages and in having this cartilage-free distal portion doubled back upon itself. The lung is clearly continuous with the trachea (Fig. 1c), and is supplied by a single, reasonably well-developed pulmonary artery that branches from the right side of the truncus arteriosus a little outside the pericardium (Fig. 1d). At the same point, the truncus arteriosus gives rise to paired systemic-carotid arteries that run cranially either side of the trachea. The single lung is interpreted as a right lung based on its arterial supply and the fact that, in contrast to aquatic caecilians, in most terrestrial caecilians the left lung is vestigial (Wilkinson, 1989). There is no trace of a left lung or left pulmonary artery. We were unable to identify the pulmonary vein in its entirety, but were able to discern its junction with the atrium just anterior to the sinus venosus sinistra, as in other lunged caecilians. The overall arrangement of the lung and its vascular supply is similar to that of other siphonopid caecilians (M. Wilkinson, pers. obs).

Additional specimens. In the field we examined the external nostrils of the caecilians we found and were initially disappointed not to find any specimen with sealed nostrils. Subsequently, after examination of the holotype of *Caecilita iwokrama*e, we reappraised our new collection and identified 11 additional specimens that are assignable to this species. Although all these specimens have more primary annuli (107–113) than the holotype

(102) the differences are not substantial and are expected to overlap with larger sample size. We found no other substantial difference in external morphology or cranial anatomy that would question our assessment of the new specimens as conspecific with the holotype of *C. iwokrama*.

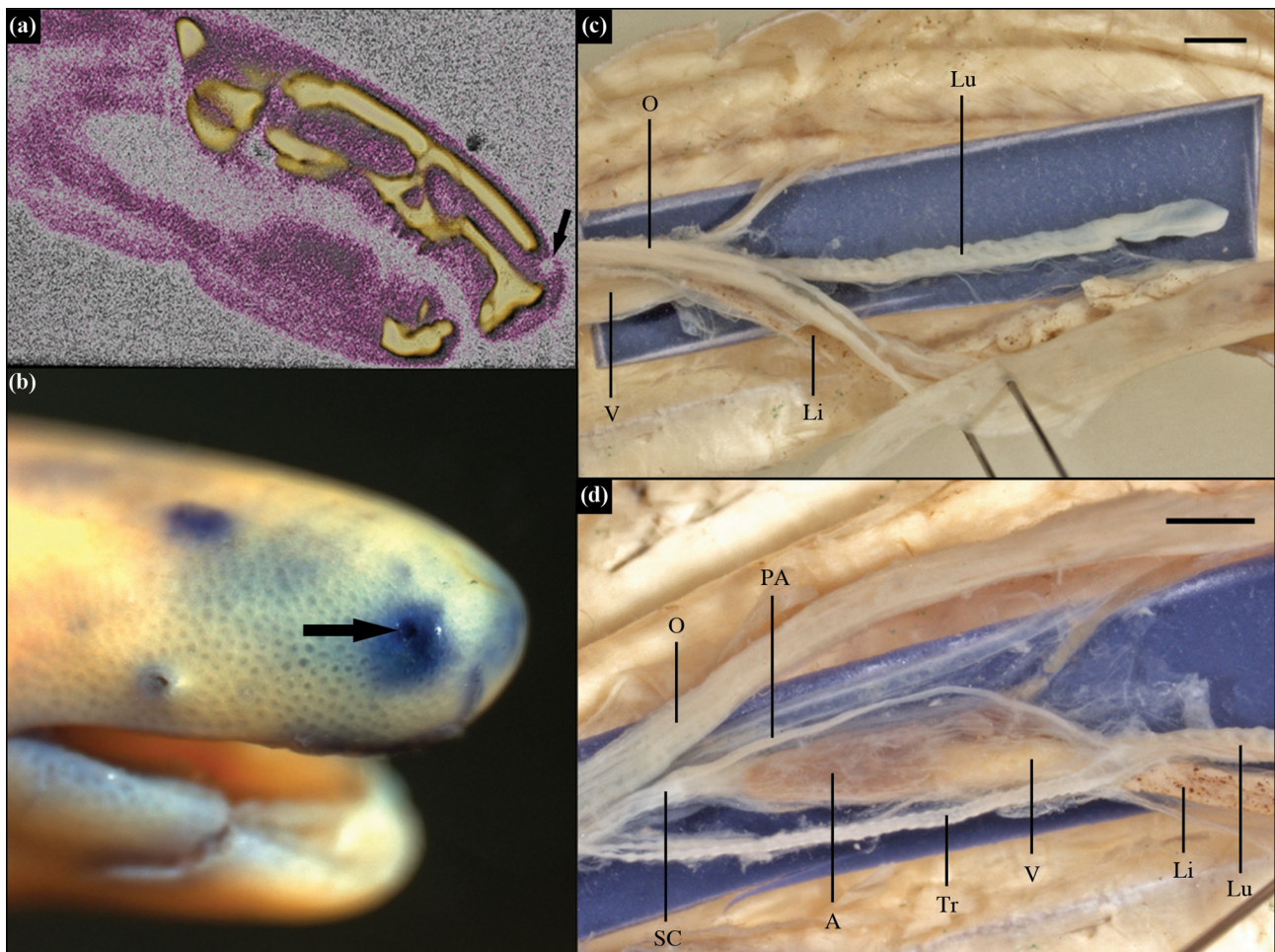


FIGURE 1. Holotype of *Caecilita iwokrama* (a) HRXCT scan with arrow highlighting the external naris in a longitudinal section, bone is pale yellow, soft tissue dark purple and the arrow c. 0.3 mm. (b) External naris indicated by arrow as revealed through temporary staining by application of a drop of methylene blue which was taken up into the nasal cavity through the external naris, arrow c. 0.5 mm. (c) Portion of coelom with the liver reflected to reveal the right lung with contrast enhanced by insertion of a dark plastic background, scale bar = 1 mm, anterior to left. (d) Portion of coelom with tissues rotated and/or reflected so as to show the dorsal surface of the heart and the pulmonary artery, scale bar = 1 mm, anterior to left. Abbreviations: Li = liver, Lu = lung, O = oesophagus, PA = pulmonary artery, SC = systemic-carotid arteries, Tr = trachea, V = ventricle.

Taxonomy. In the field, we considered the 11 caecilians we subsequently assigned to *Caecilita iwokrama* to be a species of the Neotropical genus *Microcaecilia* Taylor, 1968 (Siphonopidae). In addition to the sealed external nares that purportedly distinguished *Caecilita* from all other caecilians, Wake & Donnelly (2010) stated that *Caecilita* differed from *Microcaecilia* in having an open orbit and a small vertical keel on the terminus. Judging whether the orbit is open, closed or partially open can be difficult in caecilians without probing or dissection, especially when the eye is not visible externally. In *Microcaecilia* the orbit is mostly covered by bone (closed), but is continuous anteriorly with an open (i.e. not roofed by bone) tentacular groove. Based on our HRXCT scans we see no substantial difference between *C. iwokrama* and the type species of *Microcaecilia*, *M. albiceps* (Boulenger, 1882), in the form of the orbit that would justify the conclusion that the orbit is open in the former and closed in the latter. Dissection of our new material confirms that the orbit of *C. iwokrama* is closed, in the sense that the eye is completely covered by bone, and that there is a very short, open tentacular groove. Wilkinson & Nussbaum (2006) noted that a vertical terminal keel is present in at least some species of *Microcaecilia* and that its absence is not a diagnostic feature of the genus.

We are content that the affinities of the holotype of *Caecilita iwokrama* and of our more recently collected specimens are with *Microcaecilia*. Thus we consider *Caecilita* Wake & Donnelly, 2010 to be a junior synonym of *Microcaecilia* Taylor, 1968 and we offer the following taxonomic revision.

***Microcaecilia iwokrama* (Wake & Donnelly, 2010) new combination**

Holotype. Center for Biological Diversity, University of Guyana (CSBD) reported as number HA 1500, bearing tag MAD 2018.

Diagnosis. A *Microcaecilia* with fewer secondary annuli than all other *Microcaecilia* except *M. taylori* and *M. dermatophaga* Wilkinson *et al.*, 2013; differs from *M. dermatophaga* in having a shorter tooth row on the paired nasopremaxillary bones with fewer (<12 vs >13) teeth in total; differs from most *M. taylori* specimens in lacking a dorsal transverse groove on the first nuchal collar and in usually having fewer primary annuli (<114 vs. 113–130).

Remarks. All the new specimens were collected by digging soil with hoes, particularly under rotting wood or leaf litter or between the buttress roots of trees. We collected 13 specimens (11 subsequently preserved) of *Microcaecilia iwokrama* in an estimated total of approximately 56 person hours of digging across altitudes of c. 100–850 m. All specimens were swabbed and found to be negative in a PCR-assay for the presence of chytrid (Gower *et al.*, 2013). These findings suggests that there is a healthy population of *M. iwokrama* and that the species is reasonably common in the vicinity of the type locality. Wilkinson *et al.* (2013) recently provided a key to the species of *Microcaecilia* and a generic diagnosis that included reference to the presence of lungs (to distinguish *Microcaecilia* from *Caecilita*) that is no longer necessary.

Discussion

Wilder & Dunn's (1920) "classical" RDB hypothesis to explain the evolution of lunglessness was not seriously challenged for more than 60 years until lunglessness in plethodontid salamanders was proposed to be a consequence of reduced energetic efficiency of lung ventilation concomitant with either selection for small head size (and thus a reduced buccopharyngeal pump; Ruben & Boucot, 1989) or of a transition from aquatic to terrestrial courtship and specialisations for terrestrial feeding (Reagan & Verrell, 1991). Although these alternatives were debated without any clear conclusion in the 1990s (e.g. Beachy & Bruce, 1992; Ruben *et al.*, 1993; Bruce *et al.*, 1994) the RDB remained a plausible explanation of lunglessness in salamanders. More recently, discoveries of independently evolved lunglessness in an aquatic caecilian (Nussbaum & Wilkinson, 1995) and in a hill-stream-dwelling frog (Bickford *et al.*, 2008) were interpreted as support for the RDB hypothesis (Hutchison, 2008). Hoogmoed *et al.*'s (2011) discovery of living populations of the lungless aquatic caecilian *Atretochoana eiselti* (Taylor, 1968) within the Amazon basin provided the first concrete evidence of the environment of that species. Their discoveries falsify the speculation that this species inhabits cold, upland waters (Wilkinson & Nussbaum, 1997), but nonetheless supports the notion (Wilkinson & Nussbaum, 1997) that it inhabits fast-flowing waters where lungs could provide disadvantageous buoyancy.

To date, the only instance of lunglessness in an amphibian that cannot be plausibly explained by the RDB hypothesis is the reported lunglessness of the holotype of *Caecilita iwokrama*. Wake & Donnelly (2010) interpreted this species as terrestrial and as having evolved lunglessness in a terrestrial environment, which is clearly inconsistent with the RDB hypothesis providing a general explanation for the evolution of lunglessness. Wake & Donnelly (2010) saw no evidence from this instance of lunglessness to support either reduced buoyancy or energetic efficiency hypotheses for the origin of lunglessness and instead suggested that in this species its small-size, high surface to volume ratios, a highly vascular tongue and presumed low metabolic rate may have facilitated high cutaneous respiration and ultimately loss of the lungs for reasons not yet determined.

That the holotype of *Caecilita iwokrama* is here shown to be a member of a species of *Microcaecilia* with a well-developed lung supplied by well developed pulmonary vessels as well as internal and external nostrils similar to those of lunged caecilians is highly significant for our understanding of the evolution of lunglessness in amphibians. It removes the only putative example of an instance of lunglessness that cannot be plausibly explained by the RDB hypothesis and restores the potential of the RDB hypothesis as a general explanation of the evolution of lunglessness in amphibians.

The inaccurate claims of lunglessness and of other associated internal and external morphological features of the holotype of *Caecilita iwokrama* are surprising. Whereas the apertures in the external and internal nares are difficult, but by no means impossible, to discern with light microscopy, the lung is well-developed, readily located in the expected anatomical position, and receives a readily discerned arterial supply. Our conflicting observations demonstrate that observation reports in comparative anatomy can sometimes be misleading. Given its rarity, the discovery of any additional independent instance of amphibian lunglessness is important and any claim to have done so is substantial. We recommend, in addition to attempting thorough documentation, investigators making surprising and substantial morphological discoveries should seek opportunities to have their basic observations corroborated by independent observers, particularly when the observations challenge a widely accepted general explanation for some class of naturally occurring phenomena.

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