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A developmental staging series for the African house snake, *Boaedon* (*Lamprophis*) *fuliginosus*

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ABSTRACT

Embryonic staging series are important tools in the study of morphological evolution as they establish a common standard for future studies. In this study, we describe the *in ovo* embryological development of the African house snake (*Boaedon fuliginosus*), a non-venomous, egg-laying species within the superfamily Elapoidea. We develop our staging series based on external morphology of the embryo including the head, eye, facial prominences, pharyngeal slits, heart, scales, and endolymphatic ducts. An analysis of embryonic growth in length and mass is presented, as well as preliminary data on craniofacial skeletal development. Our results indicate that *B. fuliginosus* embryos are well into organogenesis but lack well-defined facial prominences at the time of oviposition. Mandibular and maxillary processes extend rostrally within 8 days (stage 3), corresponding to the first appearance of Meckel's cartilages. Overall, the development of the craniofacial skeleton in *B. fuliginosus* appears similar to that of other snake species with intramembraneous bones (e.g., dentary and compound bones) ossifying before most of the endochondral bones, the first of which to ossify are the quadrate and the otic capsule. Our staging series is the first to describe the post-ovipositional development of a non-venomous elapoid based on external morphology. This species is an extremely tractable captive that can produce large clutches of eggs every 45 days throughout the year. As such, *B. fuliginosus* should be a good model for evolutionary developmental biologists focusing on the craniofacial skeleton, loss of limbs, generational teeth, and venom delivery systems.

1. Introduction

In order to establish a common framework for researchers, staging series are an important and necessary precursor to studies on the developmental biology of animal species. Additionally, staging series are valuable to comparative morphologists interested in the evolution of phenotypic traits within and across clades.

Previous authors have developed classic staging series for a number of vertebrates including *Xenopus* (Nieuwkoop and Faber, 1956), mouse (Rugh, 1968; Theiler, 1972), chicken (Hamburger and Hamilton, 1951), turtle (Yntema, 1968), and a fish (Kimmel et al., 1995). Over the past decade, additional staging series have been developed for a variety of species, in part as a result of a keen interest in evolutionary developmental biology (a.k.a. evo-devo; Hall, 1992; Breuker et al., 2006) with scientists seeking to establish new model organisms. Additionally, authors analyzing temporal shifts during development (i.e., heterochrony; Smith, 2001) and timing of developmental sequences within a phylogenetic framework advocate the use of more ubiquitous events in these staging series to facilitate comparative studies (Jeffery et al., 2005; Werneburg, 2009).

Recent interest in the evolutionary development of non-avian reptiles has grown because these organisms are proving to be good models in understanding basic vertebrate evolution (Nagashima et al., 2005; Sanger et al., 2008; Wise et al., 2009). Snakes in particular have a suite of characters that interest developmental biologists, such as the lack of limbs (Cohn and Tickle, 1999), basal jaw and palatal features including generational teeth (Boughner et al., 2007; Handrigan and Richman, 2010a,b), and venom delivery systems (Vonk et al., 2008). Relative to classic model vertebrates, the development of ectotherms like snakes is slower, thus allowing researchers to observe ossification sequences for individual bones in an amniote (Haluska and Alberch, 1983; Boughner et al., 2007). Our interest in snake developmental biology was forged from a desire to understand the proximate and ultimate determinants of their trophic morphology. As limbless predators, snakes rely largely on their head and jaws to subdue and consume large prey and the remarkable success of their radiation (3000+ species) is thought to be largely due to innovations of their oral morphology (Gans, 1961; Cundall and Greene, 2000).

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To date there are a number of studies describing the embryonic development of snakes (Ballowitz, 1901; Krull, 1906; Viefhaus, 1907; Treadwell, 1962; Korneva, 1969; reviewed in Hubert, 1985). Only five studies report complete staging series from oviposition to hatch (or from gastrulation to birth for viviparous species) based on external morphology. These include two viviparous species, the Eastern garter snake (*Thamnophis sirtalis*; Zehr, 1962) and the asp viper (*Vipera aspis*; Hubert and Dufaure, 1968) plus three oviparous species, the monocled cobra (*Naja kaouthia*; Jackson, 2002), the European grass snake (*Natrix natrix*; Rupik, 2002) and the African rock python (*Python sebae*; Boughner et al., 2007). These studies have forged the way for early studies of evo-devo in snakes, but each of these species suffers from issues that make them less than ideal model organisms. For instance, obtaining embryos from viviparous species requires surgery whereas other species pose husbandry challenges such as excessively large body size, infrequent reproduction, and/or are venomous.

The aim of this study is to create a standard staging series for the post-ovipositional development of *Boaedon fuliginosus*, a non-venomous, oviparous snake with an extremely prolific reproductive capacity. We predominantly base our stages on easily diagnosed, external morphological features that reflect developmental events common to many vertebrates (Jackson, 2002; Boughner et al., 2007; Werneburg, 2009). Consequently, this work will augment our understanding of vertebrate developmental biology in general and will facilitate studies on snakes in particular.

2. Materials and methods

2.1. Material—characteristic features

The African house snake (*Boaedon fuliginosus*) is a moderate-sized (60–90 cm), non-venomous, egg-laying species that is extremely tractable as a captive animal. *B. fuliginosus* is a member of a radiation of snakes (informally designated as the 'African radiation' by Kelly et al., 2009) that is sister to the Elapidae (collectively the Elapoidea), the snake family containing the venomous and biomedically important cobras and coral snakes (e.g., de Lima et al., 2005; Nair et al., 2007). Until recently, this species was assigned to the widespread genus *Lamprophis*. However, taxonomic analyses have revealed that *Lamprophis* is polyphyletic (Kelly et al., 2009). As a result, this species was formally placed within the genus *Boaedon* along with species formerly known as *Lamprophis virgatus*, *Lamprophis lineatus*, and *Lamprophis olivaceus* (Kelly et al., 2011). The Elapoidea are a diverse group that includes species that are rear-fanged (opisthoglyphous), front-fanged (solenoglyphous, proteroglyphous), and fangless (aglyphous). As a result of its taxonomic affinities, a staging series for *B. fuliginosus* would be an important tool for comparative developmental biologists interested in the evolution of venom (e.g., Fry et al., 2008) and/or maxillary dentition (e.g., Vonk et al., 2008).

The distribution of *B. fuliginosus* spans much of the African continent, extending into the Arabian Peninsula (Branch, 1998; Kelly et al., 2011). It is adaptable, feeds on a wide range of prey, and tolerates a variety of habitats, including urban areas (hence the common name "house snake"; Branch, 1998). In captivity, *B. fuliginosus* has a voracious appetite, will readily consume frozen/thawed mice, and all life stages can be sustained on variously sized mice (hatchling *B. fuliginosus* readily take newborn mice within days of hatching while adult female *B. fuliginosus* will consume approximately three adult mice per meal). However, the most striking character, and perhaps the most important for a developmental model species, is its prolific reproductive capacity. In the laboratory, female *B. fuliginosus* will produce clutches of 5–15 eggs every 40–50 days throughout the year (maximum production in our lab was 97 eggs [8 clutches] in one year by a single female). Its taxonomic placement, oviparous reproduction, simple husbandry requirements, and prolific reproduction make *B. fuliginosus* an ideal snake developmental model.

2.2. Acquisition and incubation of eggs

Eight adult *B. fuliginosus* (6 female, 2 male) were obtained from Dr. Neil Ford of the Ophidian Research Colony at the University of Texas–Tyler in August 2008. Snakes were housed in plastic tubs (57 cm × 41 cm × 15 cm) within a temperature-controlled rack system (Boaphile Plastics, Cannon Falls, MN, USA) in a room that was maintained at 28 °C. Females were fed frozen/thawed adult mice equaling 10–20% of body mass twice weekly while males were fed 1–2 sub-adult mice twice a month. To encourage mating, males were frequently introduced to a female's cage. Reproductive behavior (Walker and Ford, 1996) was infrequently observed and few instances of copulations were witnessed. Males were generally retained in a female's cage for 5–7 days, removed, and the female was fed again. After the female digested her meal, the male would be reintroduced to her cage, usually within 5–7 days.

Once gravid, females would lose interest in feeding, which we found to be a good indicator of gravidity. We confirmed gravidity by palpation of developing follicles/eggs. Approximately 5–7 days prior to ovipositing, females would shed their skin. At this time, the water bowls were removed from the female's cage and a plastic container (21 cm × 21 cm × 7.5 cm) filled with saturated sphagnum moss (hereafter "ovipositing box") was added. Ovipositing boxes were checked daily for eggs. We typically found females coiled around a clutch in the early morning, as the majority of clutches were laid during the night. During oviposition, as the eggs exit the oviduct, a sticky substance coats the eggs, causing them to adhere to one another in a mass. By completely saturating the sphagnum moss in the nesting box, we were able to minimize egg adhesion and facilitate egg separation. Upon finding a clutch, the eggs were separated from one another, individually labeled using a pencil, and transferred to an incubator (plastic containers 26 cm × 19 cm × 8 cm, maintained at 28 ± 2 °C) filled with moist vermiculite (approximately 1:1 mixture of water:vermiculite). Time from oviposition to hatching at 28 °C was 60 ± 5 days ($N = 16$).

Six female *B. fuliginosus* produced an average of 9.2 (±2.4) eggs from 46 clutches laid over the course of two years resulting in a total of 423 eggs. Clutches were produced on average every 65 (±30) days with a minimum of 40 days between clutches. From these clutches we describe the morphology using 122 embryos in the staging series.

2.3. Collection of embryos for morphological description

Embryos were harvested at various intervals throughout post-ovipositional (p.o.) development. Through a U-shaped opening in the shell made with dissection scissors, each embryo was exposed and removed. Upon removing the embryo from the yolk it was immediately immersed in cold (4 °C) phosphate-buffered saline (PBS). Photographs were taken of the head and body morphology using a Canon EOS 5D digital camera with a 100 mm *f*/2.8 macro lens and incident light. After being photographed, embryos were sacrificed by immersing in tricaine methanesulfonate (MS222) at a dosage of 5–10 mg/egg. For more developed embryos (16 days p.o. to hatching), MS222 was administered directly into the egg prior to embryo removal. Embryos were transferred into 4% paraformaldehyde (PFA), fixed overnight at 4 °C, washed in cold PBS, and stored in 70% ethanol.

2.4. Characters scored for use in staging series

To characterize the post-ovipositional development of *B. fuliginosus,* we selected many characters used for staging other snake