Three-dimensional pelvis and limb anatomy of the Cenomanian hind-limbed snake *Eupodophis descouensi* (Squamata, Ophidia) revealed by synchrotron-radiation computed laminography

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THREE-DIMENSIONAL PELVIS AND LIMB ANATOMY OF THE CENOMANIAN HIND-LIMBED SNAKE *EUPODOPHIS DESCOUENSI* (SQUAMATA, OPHIDIA) REVEALED BY SYNCHROTRON-RADIATION COMPUTED LAMINOGRAPHY

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ABSTRACT—Cretaceous marine hind-limbed snakes are considered to be key fossils for understanding the origin and evolution of snakes. In view of the rarity of such fossils, performing new analyses on described specimens using emerging, cutting-edge techniques should bring important new insights on these forms. We investigated the three-dimensional morphology and inner architecture of the pelvic girdle and hind-limb bones of the type specimen of *Eupodophis descouensi* Rage and Escuillié, 2000, one of the three taxa for which at least one hind-limb is known, using synchrotron-radiation computed laminography (SRCL), a recently developed non-destructive technique that overcomes some of the limitations of synchrotron microtomography for flat, laterally extended objects. This experiment allowed a virtual exhumation of the second, hidden leg of the specimen. The morphology and proportions of the regressed pelvic and hind-limb bones of *Eupodophis* resemble those of the hind-limbed snakes *Pachyrhachis* and *Haasiophis*. As in *Haasiophis*, four tarsals are observed in each limb, but there are no traces of metatarsals or phalanges. Moreover, despite the presence of osteosclerosis and pachyostosis in the vertebral and the ribs of *Eupodophis*, the inner structure of its limb bones is devoid of these osseous specializations and displays a microanatomical organization similar to that of extant terrestrial lizards. This suggests that limb regression in *Eupodophis* was not due to a qualitative alteration of growth but, more likely, to a local decrease in growth rate or shortening of growth duration.

INTRODUCTION

During the Cenomanian (early Late Cretaceous), the tropical carbonate platforms of the northern and southern margins of the Mediterranean Tethys hosted the radiation of the marine hind-limbed snakes (Bardet et al., 2008). These ophidians have an elongated (0.5–1.5 m long), laterally compressed body, and (where known) a short, strongly laterally compressed tail. They all display pachyostotic vertebrae and ribs. But, above all, they are characterized by the possession of short, regressed hind-limb bones of plesiomorphic morphology (Bardet et al., 2008). Among hind-limbed snakes, only three taxa for which at least one hind-limb is known, *Pachyrhachis* Tchernov et al., 2000, and *Pachyophis* Haasiophis, 1929) have hind-limb elements preserved. The other genera (*Mesophis* Bolkay, 1925, *Pachyophis* Nopcsa, 1923, and *Similiophis* Sauvage, 1880) are represented by specimens with no preserved (or exposed) limbs. They are nevertheless considered as possible hind-limbed snakes based on other morphological features (Rage and Escuillié, 2003).

There is currently no consensus about the phylogenetic status and position of hind-limbed snakes (Rage and Escuillié, 2003). Whereas some authors consider them to be the sister group (if monophyletic) or stem group (if not) of all other snakes (e.g., Lee and Caldwell, 1998; Lee et al., 1999; Caldwell, 2000; Lee et al., 2007; Lee, 2009), others place them within macrostomial snakes, a derived group of snakes (e.g., Zaher and Rieppel, 1999; Rieppel and Zaher, 2000; Zaher et al., 2009). Because hind-limbed snakes may (or may not) represent the most primitive snakes, the study of their remains is of great significance for the question of the origin of snakes.

Despite the presence of pelvic girdle remains (lacking contact with the vertebral column) and (reduced) femora in most extant snake families (Rage and Escuillié, 2003), snakes are generally considered to be limbless squamates. The presence of reduced but fully developed hind-limb bones in *Eupodophis* offers an opportunity to further document the mechanisms responsible for limb regression within Ophidia.

The aim of the present study was to document the three-dimensional (3D) morphology and inner structure of the pelvic girdle and hind-limb bones of the type specimen of *Eupodophis descouensi*, in order to better understand their mode of regression.

Institutional Abbreviations—ESRF, European Synchrotron Radiation Facility, Grenoble, France; KIT, Karlsruhe Institute of Technology, Karlsruhe, Germany; WDC, Wyoming Dinosaur Center, Thermopolis, Wyoming, U.S.A.

Anatomical Abbreviations—A, astragalus; Ca, calcaneum; dt, distal tarsal; F, femur; Fi, fibula; Il, ilium; Is, ischium; Pu, pubis; T, tibia.

MATERIALS AND METHODS

Materials

The material investigated is the type specimen of *Eupodophis descouensi* Rage and Escuillié, 2000, discovered in the Cenomanian of Al Nammoura, Lebanon, and recorded under the

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catalog numbers WDC-C-L-N-0030A and WDC-C-L-N-0030B. It consists of two calcareous plates (part and counterpart) on which the two (ventral and dorsal) halves of the fossil specimen are preserved (Fig. 1). Whereas the right leg and associated ischium are exposed in this individual, the left half of the pelvic girdle and the left hind-limb are not.

In order to compare the inner structure of *Eupodophis* limb bones with that of four-legged extant squamates, traditional X-ray radiographs of various terrestrial lizards (*Helodermia* *suscpectum*, *Iguana iguana*, *Timon lepidus*, *Lacerta viridis*, *Tupinambis teguixin*, and numerous species of *Varanus*) have been examined.

**Methods**

The uniqueness of the *Eupodophis* type specimen completely excluded the possibility of invasive investigations. The experiment was carried out with the purpose of non-destructively imaging the three-dimensional outer and inner structures of particular regions of interest. The shape and the X-ray absorption contrast of the specimen being poorly suited for a conventional microtomographic investigation, we performed the experiment using the ESRF synchrotron. Using a third generation synchrotron source (see Kunz, 2001) instead of a conventional laboratory one to image fossil specimens allows the collection of much better quality data (Tafforeau et al., 2006). In the present case, however, microtomography was not suitable due to the shape of the specimen, composed of two broad and relatively thin calcareous plates (ca. 30 × 30 × 3 cm), because such objects engender high heterogeneity in beam transmission during object rotation in a classic computed tomography (CT) experiment, leading to complete absorption of the beam when the latter crosses the long axes of the plate. In order to overcome that limitation and to achieve data of sufficient quality for image segmentation, we employed synchrotron-radiation computed laminography (SRCL) (Helfen et al., 2005). For SRCL, the rotation axis is inclined at a known angle (typically between 15° and 45°), instead of being perpendicular to the incoming beam, as in tomography. As a consequence, the beam is never transmitted in the directions where the specimen is extended. Such a geometry leads to a rather homogeneous integral absorption by the sample during its entire rotation, but also to some missing data. SRCL is thus characterized by specific artifacts (loss of contrast and of resolution in the direction of the rotation axis), but these nevertheless are less problematic for data processing than those resulting from tomography on such extended objects. As for microtomography, the use of a synchrotron source for laminography instead of a conventional one leads to far better results, allowing higher spatial resolution, higher signal-to-noise ratio, elimination of artifacts due to polychromaticity, and the possibility of using propagation-based phase contrast (Cloetens et al., 1996; Tafforeau et al., 2006; Helfen et al., 2009).

Experiments were performed at the ESRF on beamline ID19. We used a double-crystal monochromator in Bragg geometry using a Si 111 reflection to obtain a monochromatic X-ray beam at an energy of 60 keV. The detector was a FReLoN (fast read-out low noise) charge-coupled device camera (Labiche et al., 2007) imaging a scintillating Gadox powder screen via a magnifying light–optical lens system and yielding an effective isotropic pixel size of 30.3 μm. The sample-detector distance was set to 1 m, leading to a weak edge enhancement due to phase contrast (Helfen et al., 2009) in order to obtain a better visibility of specimen structures (Tafforeau et al., 2006). The axis inclination angle was set to 30° with respect to the beam normal. For each scan, we acquired 900 projections over 360° specimen rotation with 0.7 s of exposure time. Data were reconstructed using a specific algorithm developed at KIT for laminographic reconstruction. Its principles are explained in more detail in Helfen et al. (2006). Image segmentation and visualization were performed using the software Amira, version 4.1.1.

**RESULTS**

**Pelvic Girdle**

Three pelvic bones were successfully segmented on the left side of the specimen (Fig. 2A). As in the two other hind-limbed snakes, the bones are much reduced and nearly featureless. They were identified by comparisons with the general shape of the pelvic girdles of the hind-limbed snakes *Pachyrhachis* (Lee and Caldwell, 1998) and *Haasiophis* (Rieppel et al., 2003). Because these bones are disarticulated, alternative identifications are not
impossible. The absence of sutures between these elements implies that the pelvic bones in *Eupodophis* were very loosely attached to each other, which could explain the displacement of these bones from their original position (as reconstructed in Fig. 2B).

The curved morphology of the ilium resembles that of *Haasiophis*. As it seems to narrow medially, the ilium is distinguished from that of *Pachyrhachis*, which displays an expanded medial end. There is some indication of two articular surfaces, for the ischium and the pubis, on the ventral end of the ilium. However, because of a probable displacement of this bone, there is no information about a possible sacral contact.

The ischium is very similar to that of *Pachyrhachis* in its shape and proportions with respect to the other bones (about half the length of the ilium). Its middle region is subcircular in cross-section, whereas its extremities are more expanded and flattened. A fossa on the expanded (proximal) portion of the ischium might be part of the acetabulum.

The pubis is rod-like and displays slightly expanded ends, of which the larger is probably the medial. The morphology and the relative size of this bone are roughly consistent with those of the very poorly known pubes of *Pachyrhachis* (Lee and Caldwell, 1998) and *Haasiophis* (Rieppel et al., 2003).

**Limb Anatomy**

The femur is straighter and more slender than that of other aquatic pythonomorphs, with moderately expanded ends. Contrary to what is observed in *Pachyrhachis* (Lee and Caldwell, 1998), the posterior margin is nearly straight and the anterior one is concave. The adductor crest seems to be absent, as in *Pachyrhachis*. The distal margin seems to display distinct articular surfaces for the tibia and the fibula.

Tibia and fibula are similar in length, as in *Pachyrhachis*, although the tibia is stouter as is common in reptiles (Romer, 1956). Whereas the anterior margin of the tibia appears straight, the posterior margin is concave. Conversely, the posterior margin of the fibula, which appears particularly bowed, is markedly convex and the anterior one concave. The spatium interosseum is spindle-shaped.

SRCL revealed the second (right) leg of *Eupodophis*. In each limb, four tarsal bones are observable and display the same spatial organization (Fig. 1B, D; Fig. 3). They look subcircular in shape and quite featureless. On the left limb, the astragalus and the calcaneum are in contact with, respectively, the tibia and the fibula. The astragalus is much bigger than the calcaneum, as in ‘aigialosaurs’ (Caldwell et al., 1995). The two bones are neither sutured nor fused together (whereas they are coossified in some ‘aigialosaurs’; Polcyn et al., 1999; Smith and Buchy, 2008), which might represent paedomorphosis. The two other bones are identified as the distal tarsals III (small) and IV (larger), because the presence of only these two tarsals is a common feature of the Pythonomorpha (Caldwell et al., 1995; Caldwell, 2006; Smith and Buchy, 2008). There is no trace of other distal tarsals and, contrary to what is observed in *Haasiophis* (Rieppel et al., 2003) and suggested in *Pachyrhachis* (Lee and Caldwell, 1998), there are no traces of metatarsal bones. No phalanges could be observed either.

**DISCUSSION**

**A Remark about Limb Regression**

Limb reduction, a process characterized by a decrease in the size of the limb elements, poor development of osseous structures (engendering featureless bones), and/or complete loss of
some bones (Jerez and Tarazona, 2009), is encountered in numerous squamate taxa (Greer, 1991). There appears to be a correlation between decrease in relative limb length, digit loss, and body elongation (Raynaud, 1989; Greer, 1991; Cohn, 2001; Wiens and Slingluff, 2001; Brandley et al., 2008), which probably reflects an influence of shared functional and developmental constraints on the evolution of body form in the adaptation to lateral undulation (Gans, 1975; Brandley et al., 2008).

The transition from lizard-like to snake-like body form is more or less gradual (Wiens and Slingluff, 2001), and so does not involve sudden anatomical transformations, as shown by the numerous species with intermediate degrees of limb loss and body elongation (e.g., in scincids and gymnophthalmids). However, though the correlation between body elongation and limb loss is well established, no common developmental mechanism links these specializations (Sanger and Gibson-Brown, 2004). Limb reduction seems to be caused by Hox genes. They act through the loss of axial regionalisation via homeotic changes of genes involved in the thoracic or sacral regions. This mechanism can notably explain why forelimb loss is not common in non-ophidian taxa that retain cervical vertebrae (Sanger and Gibson-Brown, 2004). It must be pointed out that it is suggested that different developmental mechanisms might be responsible for forelimb loss and hind-limb reduction in squamates (Sanger and Gibson-Brown, 2004). As for loss of digits, it appears as a developmental by-product of limb reduction. A reduction in the number of cells in the developing limb bud might engender this phenomenon (Raynaud, 1985; Wiens and Slingluff, 2001). It was previously noted that a consistent relationship between reduction in absolute limb size and loss of digits is likely to occur in anguids (Wiens and Slingluff, 2001), but the absence of distal elements in the forelimb of only one specimen of the ophidomorph *Adriosaurus*, which is not the smallest specimen assigned to that genus (Palci and Caldwell, 2007), and the absence of metatarsal bones in femora of *Eupodophis* that are of similar size to those of *Haa-
siophis*, seems to invalidate this relationship. Conversely, body elongation results from heterochronic changes in the segmentation clock (Pourquie, 2001, 2003), i.e., from molecular mechanisms acting on the timing (rate or duration) of somitogenesis in vertebrates, which increases either the total number of vertebrae or their length, because somites are the precursors of vertebrae (Jouve et al., 2000).

According to Caldwell (2003), a presacral vertebral number higher than 90, as occurs in *Eupodophis*, would be associated with complete loss of the forelimbs and severe reduction of the hind limbs. Body elongation increases the efficiency of an undulatory mode of propulsion. This mode of locomotion, for which limbs can be an obstacle to efficiency, is considered as particularly adapted to burrows or dense grass (Gans, 1975), but it also facilitates anguilliform swimming. Correlation of snake-like body form and a burrowing lifestyle has been shown by different studies (Gans, 1975; Rieppel, 1988; Caputo et al., 1995; Lee, 1998) but was not significant in anguids (Wiens and Slingluff, 2001). Loss of limbs also reduces drag and hence the energetic cost of swimming (Bejder and Hall, 2002). Such a developmental process would have thus increased performance in both burrowers and axial undulatory swimmers.

### Inner Structure of the Limb Bones

Comparison of the reconstructed microanatomical architecture of *Eupodophis* limb bones with X-ray radiographs of the same bones from extant four-legged lizard species reveals that the femur, the tibia, and the fibula of *Eupodophis* (Fig. 4) have the same microanatomical organization as those of extant terrestrial lizards. In particular, the well-developed medullary cavity and metaphyseal trabecular system of *Eupodophis* are indicative of a qualitatively normal pattern for the growth in length of the bones (endochondral growth with no inhibition of bone resorption). This suggests that limb regression in this taxon was not due to a qualitative alteration of growth but, more likely, to a global decrease in limb bones growth rate or to an ontogenetic shortening of local growth duration. Testing the latter hypotheses would require the study of cyclical growth marks in virtual sections of bone diaphyses (cf. Castanet, 1985). Further development of SRCL in a near future may allow such kind of investigations at a sub-micron scale in phase-contrast mode, which is already possible using phase contrast microradiography on teeth (Taftoreau et al., 2006; Taftoreau and Smith, 2008).

*Eupodophis* characteristically displays osteosclerosis (increase in bone inner compactness) all along its axial skeleton (A.H., pers. observ.) and pachyostosis (increase in cortical bone deposits) in most of its presacral vertebrae and ribs (Rage and Escuillié, 2000). However, this study reveals that such osseous

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**FIGURE 4.** *Eupodophis* descouensi, WDC-C-LN-0030B. 3D reconstruction of the inner cavities of the femur, the tibia, and the fibula of the hidden leg. Scale bar equals 500 µm.
specializations did not occur in the limb bones of *Eupodophis* (whereas the latter bones are affected in several ‘pachyostotic’ taxa such as *Claudiosaurus* [Buffrénil and Mazin, 1989] and some Basilosauridae [Uhen, 2004] and Choristodera [Buffrénil et al., 1990]). In aquatic squamates, pachyostosis is always found in association with osteosclerosis (Buffrénil et al., 2008; Houssaye et al., 2008), whereas the reverse is not true (Houssaye, 2008). It has already been remarked that these two specializations are generally restricted to particular bones or skeletal regions (Domning and Buffrénil, 1991), but the ultimate causes of these two specializations and the reasons for the diversity of their intra-skeletal localizations remain unclear. This study clearly shows that the limbs, like (most probably) the skull, were unaffected by these osteological specializations. It thus supports the hypothesis of a controlled—rather than diffuse—determinism for pachyostosis and osteosclerosis, probably relying on local factors, such as the local differentiation, development, and activation of osteoblasts and osteoclasts.

It is worth pointing out that secondary ossification centers can be observed in neither the proximal nor the distal epiphyses of the long bones in *Eupodophis*. On the contrary, the trabecular network seems continuous from the metaphyses up to the epiphyseal surfaces. Secondary ossification centers occur in the appendicular bones of all limbed lepidosaurians. Conversely such ossification centers are absent in the vertebral centra of both lizards and snakes (Haines, 1942, 1969).

The absence of secondary centres in the limb bones of *Eupodophis* might therefore either indicate (1) that such centers were absent in this taxon or (2) that they occurred, as in other lepidosaurians possessing limbs, but tended to fuse completely with the metaphyseal region by the end of growth. The latter process is common in small- to medium-sized lizard taxa (see Buffrénil et al., 2004, for varanid lizards). Such fusion, in a normally developed limb bone, signifies that somatic growth stops, at least locally. If secondary ossification centers actually existed in the limb bones of *Eupodophis*, it could be possible that an early fusion of these centers with the metaphyses contributed, in synergy with a global decrease in limb growth rate, to limb regression.

CONCLUSION

The use of synchrotron-radiation computed laminography (SRCL) enabled us to obtain three-dimensional data on the morphology of the regressed pelvic girdle and hind-limb bones of *Eupodophis descouensi*, which were then compared with those of the hind-limbed snakes *Pachyrhachis* and *Huastiophis*. The internal structure of the limb elements was also revealed. It notably showed two interesting features of these bones under regression: a microanatomical organization similar to that of extant lizards and the absence or complete fusion of secondary ossification centers. Moreover, it also revealed the absence of osteosclerosis in this part of the skeleton, which probably bears a functional significance. The method used in this study could be used to investigate the occurrence of limb bones in other fossil snakes assumed to have retained partial hind limbs. More generally, it demonstrates the potential of SRCL for detailed non-destructive studies of fossil specimens preserved on large flat slabs. This is all the more noteworthy and promising that this kind of preservation is fairly common in the fossil record.

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