Morphogenesis of the rudimentary hind-limb of the Glass Snake (*Ophisaurus apodus* Pallas)

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**SUMMARY**

In the legless lizard, *Ophisaurus apodus*, the hind-limb primordium appears on the caudal extremity of the Wolffian ridge at an early stage in the development of the embryo (4-2 mm long). Three somites each send an extension into this young bud and distribute cells in its mesenchymal mass of cells. An apical epiblastic ridge, appearing as a fold from the exterior, forms on the limb-bud, which at this time is flattened at the distal end. A large vessel and nerves penetrate into the bud which appears normal at this stage. Necrosis then begins, first in the ridge, then in the cells of somitic derivation and finally, when the ridge has almost disappeared, in the mesenchymal cells. The bud which has been growing up till now, starts to regress at this point. It does not, however, disappear completely as it does in the forelimb, but remains as a rod-like appendage on each side of the cloaca. Inside this structure a rudimentary skeleton takes shape. The phallic primordium which appears on the ventro-caudal aspect of the hind-limb bud is not involved in this degenerative phenomenon and continues to develop.

The arrest of development may be due to the degeneration of the apical ridge; possibly resulting from a failure of induction by the somites or to an insufficient somitic contribution to the hind-limb bud.

**INTRODUCTION**

Although many studies have been carried out on the rudimentary limbs and girdles of reptiles such as in Boïdae, Typhlopidae, Leptotyphlopidae, Aniliidae in the suborder Ophidia, and in some snake-like Sauria, embryonic studies on this subject are quite limited. Corning (1895, 1900) described a relationship between the somites and the limb primordium in lizards. He did not succeed in finding such a relation in *Anguis fragilis*, nor in *Tropidonotus natrix*. In Ophidiens, Bellairs (1950) has studied the pelvic appendage of two *Trachyboa boulengeri* embryos and one of *Python molurus*. Since 1962 Raynaud and his collaborators have done extensive studies on the morphogenesis of the rudimentary limb-buds of *Anguis fragilis* (both forelimb and hind-limb). In this legless lizard the limb-buds having once formed quickly disappear. Unlike Corning, Raynaud has found a relationship between somites and limb-buds in *Anguis*, four somites in forelimb and three somites in hind-limb. Raynaud has also studied the rudimentary hind-limb in *Tropidonotus tessellatus* (1968), in...
Python reticulatus embryos (1971–2) and very recently in Scelotes brevipes embryos.

Ophisaurus apodus, like Anguis fragilis, belongs to the family Anguidae. In this animal both forelimb and hind-limb buds appear in embryonic stage. No trace of forelimb, however, can be found in the adult. In the hind-limb area, two very small rod-like appendages (approximately 2 mm long) remain on both sides of the cloaca (Fig. 2M). Sewertzoff (1931) has described his observations on the forelimb bud of a few young Ophisaurus embryos, comparing its size with the buds in Ascalabotes fascicularis and Seps chalcides, and also on the hind-limb of some quite advanced embryos. His findings were based on a limited number of embryos; no early stages were used nor were the buds studied histologically.

As Ophisaurus can be found in abundance in north of Iran near the Caspian sea, it has been possible to carry out an extensive study of the morphogenesis and histology of the rudimentary hind-limb.

MATERIALS AND METHODS

A great number of Ophisaurus adults have been captured in their natural habitat, after having mated. They were kept in laboratory for a few months. To obtain very early stages, a certain number of individuals were dissected on different days and the eggs were extracted from the oviduct. The eggs were fixed immediately in Bouin’s fixative, and the embryos were then taken out. Some eggs were fixed immediately after laying, while others were put in an incubator for varying times from a few hours up to 6 days. The temperature was 26–27 °C or 28–29 °C, and eggs were incubated in between two sterile wet pads. Not all embryos continued their development. A great number were found dead and were discarded. The live ones were fixed. Altogether 125 embryos at different stages of development were obtained. They were all examined under a stereo-microscope. Camera lucida drawings were made and many were photographed.

Abbreviations on figures

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>a.f. or ap.f.</td>
<td>apical fold</td>
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<td>all.</td>
<td>allantois</td>
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<td>all.s.</td>
<td>allantoic stalk</td>
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<td>ao.</td>
<td>aorta</td>
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<td>a.r. or ap.r.</td>
<td>apical ridge</td>
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<td>b.</td>
<td>bud</td>
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<td>coelom</td>
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<td>chord</td>
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<td>cl.</td>
<td>cloaca</td>
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<td>c.s.d.</td>
<td>cells of somitic derivatives</td>
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<td>d.r.</td>
<td>degenerating ridge</td>
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<td>d.s.d.c.</td>
<td>degenerating somitic</td>
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<td>f.a.</td>
<td>femur anlage</td>
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<td>g.</td>
<td>ganglion</td>
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<td>l.p.</td>
<td>limb primordium</td>
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<td>m.</td>
<td>mitosis</td>
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<td>m.d.</td>
<td>mesonephric duct</td>
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<td>n.</td>
<td>necrosis</td>
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<tr>
<td>n.c.</td>
<td>necrotic cells</td>
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<td>ne.</td>
<td>nerve</td>
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<td>n.s.e.</td>
<td>necrotic somitic extensions</td>
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<td>n.t.</td>
<td>neural tube</td>
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<td>ph.p.</td>
<td>phallic primordum</td>
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<tr>
<td>r.h.l.</td>
<td>rudimentary hind-limb</td>
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<tr>
<td>s.</td>
<td>somite</td>
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<tr>
<td>s.s.</td>
<td>s.s. somites</td>
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<td>s.e.</td>
<td>somitic extension</td>
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<tr>
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Camera lucida drawings of six Ophisaurus apodus embryos, showing the successive developmental stages from pre-oviposition stage (A) up to 6 days of incubation (F). For the purpose of comparison all the embryos have been drawn at the same scale. In (D–F) the very large allantois has been removed. all., Allantois; b., bud; t., tail.
Some 20 embryos were stained in toto with Carmine. Seventy-five were embedded in paraffin for histological studies. Serial sections, 6 μm thick, were cut and stained with hematoxylin-eosin method. The sections were studied and photographed.

RESULTS

For the purpose of clarity I shall describe the observations under four headings:

Formation of the limb-bud (Fig. 1A–B).

The first indication of the hind-limb primordium appears in embryos of 4-2 mm long. In these embryos the allantois is formed and measures 0-45 mm. The cervical flexure is well defined, and the body is changing from ?-shape to C-shape. The tail makes one turn of a spiral. Approximately 65 somites are metamered. Neither the eyes nor the body are pigmented yet. The maxillary bud is just visible. The mandibular arch reaches the optic fissure. The hyoid and one of the branchial arches are formed. The body wall is still largely open to the yolk-sac.

The hind-limb primordium appears as a small swelling on the caudal extremity of the Wolffian ridge (Fig. 2A, B). No relation between somites and the limb primordium can be identified from the exterior or on the sections. Upon microscopic examination, the primordium is full of mesenchymal cells covered by an epiblast which is still quite thin (Fig. 3A, B). In more advanced embryos, however, the ventral border of the three somites behind the limb-bud is not distinct, but seems to be continuous with the bud (Fig. 2C, D). In the sections, these somites each send a process towards the bud. The processes penetrate into the base of the bud, curve towards its dorsal aspect, but do not go very far and do not reach the epiblast, as in the anterior limb-bud (M.-Z. Rahmani, 1974) (Fig. 3C). At the apex of the bud the epiblast is getting thicker, forming an apical ridge (Fig. 3F). All these phenomena take place while the eggs are still in the oviduct. In the oldest embryos of this group, a rounded swelling appears on the ventro-caudal aspect of the bud. This structure indicates the formation of

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FIGURE 2

Developmental stages of the rudimentary hind-limb in Ophisaurus apodus (before the oviposition up to 6 days of incubation) (A and B) show the first appearance of the hind-limb bud. In C, D and H the relation between two or three somites with the growing bud is recognizable. In F and G the well-formed phallic primordium can be seen. E, I and J show the apical ridge, forming an apical fold on the exterior of the bud. In K the bud has taken its final shape. The phallic primordium is well pronounced. In L a shallow groove separates the bud from the phallic primordium. At this stage the bud has taken its final definite shape. M shows part of the body of an Ophisaurus apodus adult, showing the cloacal region with a small rod-like appendage on the side of the cloacal cleft, which is the remnant of the hind-limb bud. Approx. X37/50 for A, X27/3 for B and F, and X31 for the rest except for M.
Rudimentary hind-limb of Ophisaurus phallic primordium (Fig. 2F, G, 3F, G). On examination of the sections, this swelling is seen to be filled with loose mesenchymal cells, covered by a thin epiblast (Fig. 3G).

**Growth and evolution of the limb-bud (Fig. 1C, D)**

It is during this stage that the egg is normally laid. The bud continues to grow and flattens at the distal end. The apical epiblastic ridge has formed and it can be observed from outside as a small fold (Fig. 2E, I, J). On examination of the sections, the fold looks thick, and sometimes has a sinusoid basement membrane (Fig. 3H, I, J). The somitic extensions distribute cells in the bud. These cells can be distinguished from the mesenchymal cells, between which they have penetrated as a thread or mass (Fig. 3D, E). A large vessel and nerves penetrate into the limb-bud (Fig. 3F, G, H). In the oldest individuals of this series some necrosis can be seen in the apical ridge.

**Necrosis and regression (Fig. 1E)**

The embryos belonging to this series were incubated for a few hours up to a few days. During this time the bud reaches its maximum development. The ridge, still visible from outside in younger embryos, is lost in older ones. In the sections, if the ridge is still present, it shows generalized necrosis (Fig. 4A, B, G, H), while the lateral epiblast remains intact and shows some mitosis. Necrosis can be observed in the somitic extensions and in cells derived from them (Fig. 4C, D, H). In these latter cells, necrosis appears first around the end of the nerve branch, where it penetrates into the base of the bud (Fig. 4F). From here it spreads to other cells. Between the two main centres of necrosis, i.e. the epiblast and the cells of somitic derivation, the mesenchymal cells look healthy and show a number of mitotic figures (Fig. 3J). In older embryos, when the apical ridge is almost lost, necrosis has invaded the whole bulk of the bud. Once this has happened the bud begins to regress.

The phallic primordium, larger than before, is not involved in this degenerative process and continues its development.

**FIGURE 3**

Frontal and cross-sections of developing hind-limb buds in *Ophisaurus apodus* embryos. A and B show a very young bud filled with mesenchymal cells and covered by a thin ectoderm. In C, D and E the somitic extensions leading to the bud is observed. In D and E the somitic extension has distributed cells into the bud. F, G and H show formation of an apical ridge. Phallic primordium is also observed in F and G. The apical ridge observed in H is shown with higher magnification in I. This ridge is still healthy. In J necrosis appears in the ridge, while mitosis is abundant in the mesenchyme. Note that the basement membrane of the ridge is irregular. Approx. X77 for H, X333 for D, I and J and X83 for the rest.
Establishment of the adult form of the limb (Fig. 1 F)

The embryos of this group have been incubated for more than 4 days and up to 6 days. They are much larger (7.2–8 mm long). The tail in these has made five spiral turns. The visceral arches are fused at this stage, and the cervical sinus is formed. The frontal and lateral nasal folds are fused to the maxillary process, thus forming the external narines. The very large allantois surrounds the whole embryo. The limb-bud has undergone a relative regression. No trace of apical ridge is visible either from the outside or in sections. The bud has taken the rod shape seen in adult individuals. A shallow groove separates it from the phallic primordium (Fig. 2 L). In sections from some younger embryos, some necrotic cells can be observed still, but the majority have already disappeared. In embryos which have been incubated for more than 5 days, the bud is filled with normal mesenchymal cells, inside which a clump of more condensed cells is observed (Fig. 4 I). The epiblast covering the bud is thin at this time. The phallic primordium has continued to grow (Fig. 4 H). It is formed by a mass of mesenchymal cells covered by a thin epiblast.

DISCUSSION

The course of hind-limb development in Ophisaurus is as follows. The primordium of the hind-limb appears on the caudal extremity of the Wolffian ridge by a condensation of somatopleural cells. As no great number of mitoses are observed at this time, it seems likely that the cells have migrated to this point. Once the primordium becomes visible from the exterior, three somites send an extension each towards this young bud. The extension penetrates into the bud, bending toward its dorsal aspect. These extensions seem to distribute cells inside the bud, thus contributing to its substance and its growth. Nevertheless, this contribution is much more restricted than in the forelimb. (In the forelimb of Ophisaurus four to five somites penetrate into the cranial extremity of the Wolffian ridge, inducing the formation of the bud and then contributing to its substance.) An apical ridge which appears as a fold from the outside forms on the limb-bud.

FIGURE 4

Frontal and cross-sections of the hind-limb bud in Ophisaurus apodus embryos, showing the degenerative process leading to the regression of the bud. Necrosis has invaded the apical ridge in A, B, G and H. In H cells of somitic derivatives and mesenchymal cells are also involved, but the mesenchyme in the phallic primordium is not affected at all. E shows the three somites which send an extension into the bud (extensions cannot be seen on this section). In C necrosis can be observed in two somitic extensions and D shows necrosis in cells of somitic derivatives. In F necrotic cells are observed around the extremity of the nerve. I shows a quite advanced bud in which necrotic cells have disappeared. The clump of condensed cells represents the skeletal anlage, which is probably the femur. Approx. X77 for E, X333 for B, C and F and X83 for the rest.
The ridge does not always look quite regular and sometimes has a sinusoid inner border. A large vessel and nerves penetrate into the bud. So far, the bud does not differ greatly from those observed in other reptiles with normal limbs. At this point, necrosis starts almost simultaneously in the apical ridge and in the cells of somitic derivation. In these latter cells necrosis appears first around the extremity of the nerve, where it penetrates the bud, then goes farther and even invades the somitic extension itself.

While the process of necrosis rapidly invades the two areas mentioned above, the mesenchymal cells continue proliferating, and thus the bud continues to grow. When the ridge is almost lost, the mesenchymal cells are also invaded by necrosis, and the bud partially regresses. On the sixth day of incubation, almost all traces of necrotic cells have disappeared. No apical epiblastic ridge is visible and a healthy thin epiblast covers the bud. In sections, homogeneous mesenchymal cells are observed, inside which a more condensed mass of cells can be seen. In certain sections, it appears to have the shape of a femur and is very likely the anlage of the rudimentary skeleton as observed by Sewertzoff (1931). He found rudiments of a stylopodium, a zeugopodium, a tarsus and a phalanx in the rudimentary hind-limbs of a few advanced embryos and in adult Ophisaurus.

Several points deserve discussion. First, what is the reason for the arrest of further development, and the partial regression of the hind-limb of Ophisaurus? Since the investigations of Saunders (1948) and Zwilling (1956) a tremendous amount of research has been focused on the necessity and importance of the apical ridge for normal and complete development of the limbs in Amniota, and its role in the whole developmental aspect of the bud, such as proliferation, differentiation, and even necrosis of the mesenchymal cells, which form the bulk of the limb-bud. Saunders (1948) has shown that if the epiblastic ridge of a wing-bud of a 3-day-old chick embryo is removed, the distal parts of the wing are not formed.

Although the leading role of the whole epiblastic cover of the limb-bud in general, and the apical ridge in particular is fully accepted, different authors give different explanation for the mechanism of their action. Zwilling (1961, 1966) believes that the ridge plays the role of a mesoderm-dependent outgrowth inductor on the subjacent mesenchyme. Janners and Searls (1971) consider the ridge as an outgrowth inducer in the sense that it permits growth in appropriate directions and thereby shapes the growing mesoblast. Amprino (1965) and Amprino & Ambrosi (1973), on the other hand, suggest that the whole ectodermal cover of the limb-bud and the ridge could have a modelling role on the development of the limb, by acting as a limiting and protective cover of the vascularized mesoderm. They do not consider the apical ridge to be a special structure, but the result of proximo-distal sliding and packing of the ectodermal cells on the apex of the limb-bud.

In Ophisaurus it seems likely that the arrest of development of the hind-limb
Rudimentary hind-limb of Ophisaurus

bud is due to degeneration of the apical ridge. Necrosis appears in cells of somitic derivation almost at the same time as in the ridge and later invades the whole bulk of mesenchymal cells. For a short while, mitosis compensates for cell degeneration, but later relative and actual regression of the limb-bud is observed. However, the bud does not regress completely, and a rudiment is left in the adult. Similar observations have been reported for Anguis fragilis, in contrast to Lacerta viridis, in which the limbs undergo normal development (Raynaud, 1962 onwards). If, as suggested by Amprino and Ambrosi, the apical ridge forms by sliding of the ectodermal cover of the limb-bud, then this process has to be arrested when necrosis begins in the ridge in Ophisaurus. After the ridge has completely disappeared, a thin healthy ectoderm covers the limb-bud evenly, and no trace of apical ridge can be observed.

Secondly, what is the factor which determines the necrosis in the ridge? Murillo-Ferrol (1963, 1965), Kieny (1969, 1971) and Pinot (1969, 1970) have demonstrated the primary inductive function of the somite in the limb development. Raynaud and his collaborators since 1962 have devoted many studies to this problem in different reptiles with normal, reduced, and absent limbs. As a result of these long investigations, Raynaud found a direct relationship between the number of somites which send an extension into the limb-bud and the degree of evolution of the latter. My own investigations on the forelimb of Ophisaurus are in agreement with Raynaud’s results. In lizards with normal hind-limbs, four somites penetrate into the hind-limb bud (van Bemmelen, 1888, 1889). Raynaud has found only three somites establishing such a relation in the legless lizard, Anguis fragilis. This is exactly the same in Ophisaurus. Raynaud has assumed that the diverse degree of reduction of the limb in reptiles is the consequence of formational or functional deficiency of the somitic extensions penetrating into the limb-bud. If this assumption is proved, it would be valid for Ophisaurus as well.

RESUME

Chez l’embryon de lézard apode, Ophisaurus apodus (embryon de 4,2 mm long), l’ébauche du membre postérieur apparaît sur l’extrémité caudale de la crête de Wolff. Le jeune bourgeon ainsi formé reçoit trois prolongements provenant de trois somites. Ces prolongements essaient des cellules parmi les cellules mesenchymateuses du bourgeon. Une crête épiblastique apicale apparaît sur l’extrémité distal du bourgeon qui est au stade de jeune palette. Cette crête se présente vers l’extérieur sous forme d’un bourrelet. Un large vaisseau sanguin et des nerfs pénètrent dans ce bourgeon. Quand un bourgeon quasi-normal est formé, la nécrose apparaît d’abord dans la crête et les cellules dérivées des somites, puis gagne les cellules mesenchymateuses, lorsque la crête est presque disparue.

Le bourgeon qui a continué de croître, subit alors une regression partielle, et persistera sur les deux côtés du cloaque comme une appendice en forme d’un petit bâtonnet. A l’intérieur de cette appendice l’ébauche d’un squelette rudimentaire prend naissance.

L’ébauche phallique apparaît sur le bord ventro-caudal du bourgeon n’est pas atteint par le processus de la nécrose et continue son développement.

L’arrêt du développement du bourgeon peut-être attribué à la dégénérescence de la crête apicale; cette dégénérescence pourrait être la conséquence d’une manque de contribution des cellules somitiques ou une déficience d’induction somitique ou enfin les deux.
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