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Chapter 3: Evolutionary Origin and Development of Snake Fangs


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Many advanced snakes use fangs — specialised teeth associated with a venom gland — to introduce venom during prey capture or defence. Various front- and rear-fanged groups are recognised, according to whether fangs are positioned anterior (e.g. cobras and vipers) or posterior (e.g. grass snakes) in the upper jaw. A fundamental controversy in snake evolution is whether or not front and rear fangs share the same evolutionary and developmental origin. Solving this controversy would point to a major evolutionary transition that underlies the massive radiation of advanced snakes, and to the underlying developmental events. We examine this issue by visualising the tooth-forming epithelium in the upper jaw of 96 snake embryos, covering eight species. We use the *sonic hedgehog* (*shh*) gene as a marker, and reconstruct the development in 3D in 41 of these. Here we show that front fangs develop from the posterior end of the upper jaw, and we reveal a striking similarity in morphogenesis between front and rear fangs that argue for their homology. In front-fanged snakes, the anterior part of the upper jaw lacks *shh* expression, and ontogenetic allometry displaces the fang from its posterior developmental origin to its adult front position — suggesting a posterior evolutionary origin. In rear-fanged snakes, the fangs develop from an independent posterior dental lamina and retain their posterior position. In light of our findings, we suggest a possible new model for the evolution of snake fangs; a posterior sub-region of the tooth-forming epithelium became developmentally uncoupled from the remaining dentition. This would have allowed the posterior teeth to evolve independently and in close association with the venom gland, becoming highly modified in different lineages. This developmental event could have facilitated the massive radiation of advanced snakes in the Cenozoic era, resulting in the spectacular diversity of snakes seen today.
Many advanced snakes (Caenophidia, sensu ref. 22) use venom, with or without constriction, to subdue their prey\textsuperscript{5,107}. Their venom-delivery system includes a post-orbital venom gland associated with specialised venom-conducting fangs\textsuperscript{108}. Fangs can occupy various positions on the upper jaw, but are always located on the maxilla and never on any other tooth-bearing bone\textsuperscript{120} (Fig. 6C). Viperidae (vipers & pitvipers), \textit{Atractaspis} (Lamprophiidae, sensu ref. 22), and Elapidae (cobras and relatives) have tubular front fangs (Fig. 6B,C). The remaining lineages are all non-front-fanged and called either ‘non-fanged’ (no distinguishable enlarged posterior tooth) or rear-fanged\textsuperscript{107,120} (Fig. 6B,C). Rear fangs can be solid, slightly or deeply grooved, but never tubular\textsuperscript{120}.

There has been active debate concerning the evolutionary origin of these different fang types, and their relationships to the simple, unmodified teeth of non-fanged\textsuperscript{121} basal snakes\textsuperscript{107,109-114,121} such as pythons and boas (Boidae). Proposed hypotheses include: (i) front-fanged snakes form a monophyletic group and their fangs are derived from rear-fangs\textsuperscript{113,122,123}; (ii) elapid fangs are derived from front teeth and viperid fangs from rear fangs\textsuperscript{124,125}; (iii) elapid and viperid fangs are both derived from rear-fangs\textsuperscript{112}; and (iv) front and rear fangs have independent origins\textsuperscript{107}. Establishing the origin and evolutionary transformation series between these dentition types requires a robust phylogeny to map the characters onto. Since recent molecular phylogenies of caenophidians place the front-fanged Viperidae as relatively basal and the front-fanged Elapidae as more recently derived\textsuperscript{1,107} (Fig. 6A), the current evidence seems to support the ‘independent-origin’ hypothesis\textsuperscript{107}.
Figure 6 | Adult maxillary dentition mapped onto a molecular snake phylogeny to show relative positions of the various fang types. a, Phylogeny from ref. 16. b, c, Adult skulls (Supplementary Table 4): lateral views (b); palate, schematic ventral views (c; maxilla coloured, fangs circled). Asterisks indicate species studied by electron microscopy (Supplementary Fig. 5, Supplementary Table 3). The evolutionary changes leading from an unmodified maxillary dentition to the different fang types in advanced snakes are indicated at the nodes: (1) continuous maxillary dental lamina, no specialized subregions—ancestral condition for advanced snakes; (2) evolution of posterior maxillary dental lamina—developmental uncoupling of posterior from anterior teeth; (3) starting differentiation of the posterior teeth with the venom gland; (4) loss of anterior dental lamina and development of front fangs.
To date, this issue has not been examined using a molecular developmental approach, not least because of the difficulty of obtaining snake embryos of all the different species. Development of fangs and venom glands has been studied before in vipers 126,127, Natrix 128,129 (Natricidae), Spalerosophis 130, Thamnophis and Telescopus 106,108 (Colubridae). Those morphological studies identified a common primordium of the venom gland and fangs 106,126,128, but did not visualise the odontogenic band (tooth-forming epithelium: a band of epithelial tissue that invaginates and forms a dental lamina). Therefore, no conclusions could be drawn about the origin and evolutionary transformation series of the fangs.

Here, we have carried out in situ hybridisation of the sonic hedgehog (shh) gene in 96 snake embryos of multiple stages, and reconstructed the development of the maxillary dentition in 3D in 41 of these through serial sections. We use nine advanced snake species — comprising two front-, one rear- and one ‘non-fanged’ lineage. As outgroup we included the non-fanged waterpython (Liasis mackloti: Boidea), which is basal to advanced snakes (Fig. 6). Shh is expressed in the odontogenic band in different vertebrate species 115-117. By visualising this band, we aim to find evidence for the ancestral condition of the maxillary dentition. A list of all material studied can be found in Supplementary Tables 1-4. We map our characters onto the recently published, robust molecular phylogeny of advanced snakes obtained by Vidal et. al. 2007 (ref. 22, and Fig. 6A).

In the waterpython (L. mackloti), shh expression reveals one continuous maxillary odontogenic band (Fig. 7a). As confirmed by serial sections of embryos ranging from young to old, this band invaginates to form one dental lamina — a single continuous, invaginating epithelium that will develop a row of teeth (Fig. 8a & Fig. 9j-l). This is consistent with a recent morphological study of Python sebae 131. The odontogenic band, with its associated lamina, appears along the entire rostrocaudal extent of the upper jaw — from the premaxilla to the mandibular articulation (Fig. 7a). This suggests that the ancestral condition for the maxillary dentition of advanced snakes is one dental lamina that appears along the entire rostrocaudal extent of the upper jaw, lacking specialised sub-regions.
The early odontogenic band in the non-front-fanged grass snake *Natrix natrix* (Natricidae) and rat snake *Elaphe obsoleta* (Colubridae) is similar to that of the waterpython (Fig. 7c & Suppl. Fig. 2f,i). However, we show for the first time that there are two dental laminae which invaginate separately (Fig. 8b,e & Fig. 9a-c & Suppl. Fig. 8e-g) and fuse during development (Fig. 9c & Suppl. Fig. 3h-i). The anterior lamina bears only teeth (Fig. 9c & Suppl. Fig. 3f) and is similar in development to the one in the waterpython (Fig. 9j,k&l). The posterior lamina, however, bears teeth and forms the common primordium with a post-orbital gland (Fig. 8b & Fig. 9b-c & Suppl. Fig. 3i). These develop into the rear fangs and venom gland in the grass snake, and probably represent the first differentiation of the posterior teeth with a venom gland in the rat snake. The latter observation is consistent with a recent MRI and histology study showing the presence of a small gland in rat snakes. To verify that the anterior and posterior dental laminae are truly developmentally independent, we ablated the primordium of the anterior lamina in isolated developing upper jaws of the dice snake *Natrix tessellate* (Suppl. Fig. 3m-o). After cultivation under the yolk sac membrane, we found that the posterior lamina, with its venom gland and fangs, developed normally in the absence of the anterior lamina (Suppl. Fig. 4), showing that they are developmentally independent.
**Figure 7** | *Shh* expression in the embryonic snake palate, showing the posterior developmental origins of front fangs. a–d, Palate, ventral view: top, anterior; scale bar, 0.5 mm; dotted lines, upper jaw (posterior margin of premaxilla to attachment of the mandible); boxes, schemes of maxillary odontogenic band (purple, *shh* expression; grey, no *shh* expression). Positions of fangs in b–d were identified histologically (Fig. 3, Supplementary Fig. 3). The odontogenic band in the front-fanged species is located posterior in the upper jaw (b, d). In the non-fanged outgroup (a) and the rear-fanged *Natrix* (c), the odontogenic band extends along the entire upper jaw. f, fang; mx, maxillary odontogenic band; pa, palatine odontogenic band; pt, pterygoid odontogenic band. e, Ontogenetic allometry in the fang in the front-fanged *Causus* displaces the fang along the upper jaw (Supplementary Figs 5–9, Supplementary Tables 5–9). Scale bars, 1 mm. We note the change in relative size of the upper jaw subregions: i, anterior; ii, fang; iii, posterior. d.a.o., days after oviposition.
Figure 8 | Sections of the shh in situ hybridizations of the embryonic upper jaw in five snake species, showing the posterior and anterior dental laminae. a–c, e–f, Sagittal sections, anterior to the left, of L. mackloti (Boidae) 22 d.a.o. (a), N. natrix (Natricidae) 22 d.a.o. (b), Calloselasma rhodostoma (Viperidae) 8 d.a.o. (c), N. natrix 22 d.a.o. (e), Naja siamensis (Elapidae) 23 d.a.o. (f). d, transverse section, medial to the left, of Trimeresurus hageni (Viperidae) 8 d.a.o. The posterior maxillary dental laminae in b and e are similar in morphogenesis to the maxillary dental laminae in all front-fanged species examined (c, d, f; see also Fig. 4). Arrowheads, shh expression; amdl, anterior maxillary dental lamina; dr, dental ridge; e, eye; f, fang; mdl, maxillary dental lamina; pa, palatine dental lamina; pmdl, posterior maxillary dental lamina; t, tooth bud; vd, primordium of venom gland; scale bars, 300 mm.

In the five front-fanged species examined (Viperidae and Elapidae), the maxillary odontogenic band is found in the posterior part of the upper jaw (Fig. 7b,d & Suppl. Fig. 2a,d,g). There is no shh expression nor dental lamina in the anterior region (verified by histology, data not shown). By contrast, in the waterpython, grass and rat snake, the odontogenic band and associated dental laminae appear along the entire rostrocaudal extent of the upper jaw. We find that, during development, the ‘rear’ fang is displaced to its adult ‘front’ position by ontogenetic allometry (Suppl. Figs. 6 & 7 and Suppl. Table 6 for statistical analyses); suggesting a posterior evolutionary origin of the front fangs. Histology shows that although the odontogenic band invaginates normally and forms one dental lamina (in contrast to the non-front-fanged snakes examined above), in all front-fanged species the fangs develop from the posteriormost part of this lamina and there are no developing teeth in the anterior part (Fig 8f & Fig. 9d-
h). This apparently toothless part of the dental lamina has been described before only in *Vipera aspis* (Viperidae) and termed the ‘dental ridge’\(^{128}\). We show it here for the first time in Elapidae (Fig. 8f & Suppl. Fig. 3j-l). The fact that viperids and elapid snakes share; (i) the ‘dental ridge’ and (ii) a posterior developmental origin for their front fangs is interesting, since they are phylogenetically not closely related (Fig. 6A).

**Figure 9** (page 34) Schematic three-dimensional reconstructions showing the similarity in morphogenesis between the rear and front fangs. Derived from serial sections (Fig. 8, Supplementary Fig. 3); materials analysed are listed in Supplementary Tables 1, 2, 4. Left-hand side of the upper jaw is depicted, and only epithelial components are shown. Purple, *shh* expression; grey, tooth buds; green, unspecialized maxillary dental lamina; orange, specialized maxillary dental lamina that bears fangs. The specialized dental lamina is dilated into a bifurcated epithelial sac, the lateral part giving rise to the venom duct and venom gland by growing rostrad (see also Fig. 3b–d, f), then turning caudad to reach the post-orbital region (as previously described for vipers\(^{126,127}\), *Natrix*\(^{128,129}\) and *Spalerosophis*\(^{130}\). In *Elaphe obsoleta* (a–c) and *Natrix natrix* (data not shown), fangs develop rostrally and caudally alongside the base of the venom duct; in *Naja siamensis* (d–f) and *Trimeresurus hageni* (g–i) the rostral part regresses, remaining visible only as the dental ridge, whereas in b and c this part bears fangs and fuses with the anterior dental lamina. The unspecialized dental lamina in *E. obsoleta* (a–c) and the outgroup *Liasis mackloi* (j–l) starts developing anterior and grows caudad.
Because Elapidae and Viperidae do not form a monophyletic group (Fig. 1A), the ‘dental ridge’, the posterior developmental origin of the fangs, and the allometric growth in both lineages may still reflect convergent evolution. However, our 3D reconstructions reveal that there is a striking similarity in morphogenesis of all front and rear fangs examined (Fig. 9b-i), despite the large variation in adult morphology. The toothless ‘dental ridge’ seen in elapids and viperids is similar to the part of the posterior dental lamina that fuses with the anterior dental lamina in the grass snake and the rat snake (Fig. 9). Although developmental similarity is not conclusive proof of structural homology, this is especially interesting in light of the posterior developmental origin of the front fangs in both elapids and viperids mentioned above. These results are difficult to reconcile with the ‘independent-origin’ hypothesis, but are consistent with the hypothesis that elapid and viperid front fangs, and the posterior dental lamina in non-front-fanged snakes, represent homologous structures.

Our results suggest a possible model for the evolution of snake fangs. A posterior sub-region of the ancestral tooth-forming epithelium became developmentally uncoupled from the remaining dentition, resulting in a posterior and anterior dental lamina that are developmentally independent (Suppl. Fig. 1). This condition is retained in the non-front-fanged snakes, such as the grass and rat snake. This model would imply that the front-fanged elapids and viperids have independently and secondarily lost the anterior dental lamina (Fig. 6), which is supported by the lack of shh expression anterior in their upper jaws.

Since obtaining developmental data for each non-front-fanged advanced snake lineage is impracticable, convergence cannot be ruled out completely. We have, therefore, examined the adult maxillary tooth morphologies through scanning electron microscopy in the waterpython, the grass and rat snake, and a further wide range of non-front-fanged advanced snake species (Fig. 6A). We aimed to find differences in the maxillary dentition, which might suggest the presence of two maxillary dental laminae in additional lineages. Our results show, indeed, that there is a consistent difference in anterior versus
posterior tooth morphologies in additional advanced snake lineages (Suppl. Fig. 5d-x, and Suppl. Table 3). In contrast, the maxillary teeth from the Boidae do not show a morphological difference between anterior and posterior teeth (Suppl. Fig. 5f). These results suggest the possible presence of two dental laminae in other non-front-fanged advanced snake lineages, and provides additional support to our proposed model.

The developmental uncoupling of the posterior from the anterior tooth region represents a possible mechanism that could have allowed the posterior teeth to evolve independently and in close association with the venom gland. Subsequently, it could became modified and form the fang-gland complex — an event which underlies the massive radiation of advanced snakes during the Cenozoic era.
Methods

Snake embryos. Snake eggs and embryos were acquired in accordance with local and international regulations from European and Australian breeders and zoos. Eggs were incubated at 30°C and embryos fixed in 4% paraformaldehyde in PBS at 4°C overnight. They were dehydrated through graded methanols and stored at -18°C.

In situ hybridization. The RNA probe is based on the partial PCR product of SHH using the cDNA of a 1-day-old Rhombic Night Adder (Causus rhombeatus) embryo as template (GeneBank accession number EU236145). Hybridization was performed according to standard protocols. In all species examined, the odontogenic band (tooth-forming epithelium) always expressed shh (Fig. 2 & Suppl. Fig 2). This shows that, as in other vertebrate groups, shh is also a marker for odontogenic epithelium in snakes. A list of embryos studied can be found in Suppl. Table. 1.

Histology. Embryos where dehydrated through ethanol or methanols, cleared in HistoClear or tetrahydronaphthalene, and embedded in paraffin. Sections were cut at 5-7 microns and counterstained using Neutral Red or H+E. A list of embryos studied can be found in Suppl. Table. 2.

3D modeling. Schematic 3D models were drawn from analyses of the serial sections of the embryos using a Nikon Eclipse E800 microscope. All models were drawn using Adobe Illustrator and Adobe Photoshop.

Scanning electron microscopy. The maxillary bone on one side was dissected out of the specimen, allowed to dry and mounted on a stub, using double-sided tape, with teeth pointing upwards. Specimens were sputter-coated with gold, and examined using a JEOL JSM-T300 scanning electron microscope, at an acceleration voltage of 15kV. A list of specimens examined with their museum numbers can be found in Suppl. Table. 3.
Ablation experiment. Was performed as previously described 133.

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