Spatial and temporal pattern for the dentition in the Australian lungfish revealed with sonic hedgehog expression profile

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We report a temporal order of tooth addition in the Australian lungfish where timing of tooth induction is sequential in the same pattern as osteichthyans along the lower jaw. The order of tooth initiation in *Neoceratodus* starts from the midline tooth, together with left and right ones at jaw position 2, followed by 3 and then 1. This is the pattern order for dentary teeth of several teleosts and what we propose represents a stereotypic initiation pattern shared with all osteichthyans, including the living sister group to all tetrapods, the Australian lungfish. This is contrary to previous opinions that the lungfish dentition is otherwise derived and uniquely different. *Sonic hedgehog* (*shh*) expression is intensely focused on tooth positions at different times corresponding with their initiation order. This deployment of *shh* is required for lungfish tooth induction, as cyclopamine treatment results in complete loss of these teeth when applied before they develop. The temporal sequence of tooth initiation is possibly regulated by *shh* and is known to be required for dentition pattern in other osteichthyans, including cichlid fish and snakes. This reflects a shared developmental process with jawed vertebrates at the level of the tooth module but differs with the lack of replacement teeth.

Keywords: tooth development; lungfish dentition; sonic hedgehog; osteichthyan stereotype; cyclopamine inhibition; *pitx2* expression

1. INTRODUCTION

On each side of the jaw of all vertebrate dentitions the iterative initiation of teeth and the left–right mirror image on each dentate bone provide an appropriate system to study development of craniofacial symmetry in the vertebrate body plan (Smith 2003). Also the way in which the iterative pattern along the jaw is regulated could be compared within osteichthyans to establish any common developmental parameters that might be conserved in evolution. The Australian lungfish *Neoceratodus forsteri* is the closest extant sister group to tetrapods and fills the phylogenetic gap between these and all other osteichthyans (Cloutier & Ahlberg 1996). However, the arrangement of the dentition in lungfish is quite unlike most others in the total osteichthyan clade and it has been considered as so unique that even the marginal row of teeth does not relate to that of any jawed vertebrate, nor especially to those of tetrapods (Kemp 2002). A previous analysis of developmental plasticity in early dipnoan dentitions (Ahlberg *et al.* 2006) posed the question of how the stereotypic osteichthyan package had been deconstructed to become so different in dipnoans. To achieve some understanding of how development might be modified

through evolution, we have compared development of the dentition in *Neoceratodus*, the most primitive of the three living lungfish, with the stereotypical dentition patterning for osteichthyan fish. The only opportunity to find a potential common pattern is by observing the earliest stages of tooth initiation of the marginal teeth on the lower jaw. Here, teeth are situated directly above Meckle's cartilage as no dentary bone forms, and are labial to the lingual tooth plate. They are the only series of teeth that may be conserved from the typical osteichthyan marginal dentition, albeit during a transitory phase, formed early in the embryo and functional during hatchling status only. In *Neoceratodus* adults, this marginal tooth row is lost completely, while further tooth addition to the typical and unique paired pterygoid and prearticular tooth plates of upper and lower jaws continues through early growth. Our focus on regulation of the dentary tooth row is because lungfish have a unique dentition of paired upper and lower dental plates in which all the generated teeth are retained without loss through serial replacement. This is a form of statodont dentition (all teeth retained throughout life) contrasting with the continuously replacing dentitions of most jawed vertebrates. It is a fascinating question to ask how this unique dentition could have evolved from one in which tooth addition is integrated with controlled tooth replacement as the stereotypic pattern present in osteichthyans. This loss of tooth replacement in lungfish leaves only the process of regular tooth addition at new sites at the extremities of the tooth rows.

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2008.1364> or via <http://journals.royalsociety.org>.

Figure 1. (Caption opposite.)

We have targeted the initial stages of the timing of the dentary tooth pattern in skeletal preparations and the process of its potential regulation by *in situ* hybridization of genes known to be involved in commissioning teeth. In both, viewed as whole mounts of *Neoceratodus* larvae, we were able to detect not only the time order but also a discernable difference in timing of tooth formation along the jaw. This was defined by critically small time differences in the expression of *Nfshh*, chosen as one gene conserved within osteichthyans for the initiation stage of tooth development and its early morphogenesis

(Fraser *et al.* 2004). Both *shh* and *pitx2* have been found to be important genes in commissioning the dentate regions of the oral epithelium in osteichthyans and for tooth initiation in both mice and teleost fish (Cobourne *et al.* 2004; Fraser *et al.* 2004). In the trout, spatially distinct loci with different times of *Omslh* expression occurred in the dental epithelium with expression response in the subjacent mesenchyme shown by *Ombmp4* (Fraser *et al.* 2006b). We therefore, investigated in detail the expression of *shh* in *N. forsteri* larval stages, together with *pitx2* (see the electronic supplementary material), selected for the key

Figure 1. (*Opposite.*) Early tooth development in the lower jaw of *N. forsteri*. Comparison of hatchlings from stages 40–52 as whole mount skeletal preparations viewed in differential interference microscopy (DIC) and drawings of each stage. Stained with Alcian blue for cartilage and Alizarin red for calcified bone and dentine; sections with Mallory's trichrome, turquoise connective tissue, dentine and cartilage, red for calcified dentine and bone. (a) Stage 47, a 7 μ m section through two dentary teeth with bone of attachment (arrow head) and protogerm for adjacent tooth site (arrow) above Mc; inset is sequential section of right dentary tooth. (b) Stage 43, dentine of a young tooth, with cap of dental epithelium from yolk granule containing endoderm and papilla of ectomesenchyme containing melanin granules, and differentiated odontoblasts. (c) Lingual view, midline of stage 42, all tooth cones are clear against Mc; larger cones (pa.t) are teeth above paired prearticular bones (pa.b) meeting at the symphysis; anterior to this is the single symphyseal tooth (sy) of the marginal row with both sides of dentary teeth in jaw position 2 (d2) and tip only of the newest tooth at third position (d3); see drawing in (i) for entire left side at stage 42 and (h) stage 40 when only the symphyseal tooth has formed, with three prearticular teeth and new fourth tooth on the left (pa4). (d) Stage 45, lingual view, right side of the marginal set in which the tooth in position 1 is a small tip (d1), see drawing in (j) showing only the marginal teeth of the left side, with bone of attachment of the first teeth to form (ba, sy, d2, d1). (e) Stage 48, antero-lateral view of dentary teeth with fourth tooth (d4) added with bone and all teeth now joined by the bone of attachment (see stage 47 in (k) and stage 50 in (i), with fifth tooth added d5). (f,g) Control and experimental embryos (42C6/42E7) at stage 47 after treatment at stage 42 with cyclopamine, or ethanol without cyclopamine; see drawing in (k) at stage 47 of the left side with the fourth dentary tooth added posterior to the first three, these joined by the bone of attachment. (h–m) Drawings taken from the skeletal whole jaw mounts as in (c–e): (h,m) show both left and right, (i–l) are left side only, with (j,k) view of the dentary teeth alone, the youngest teeth are cones only, the older have first attachment bone, and the oldest are joined by extensive bone of attachment. Colour code: red is the pioneer second dentary tooth, yellow is dentary and orange symphyseal tooth. Abbreviations: ba, bone of attachment; d1–6, first six dentary teeth; Mc, Meckle's cartilage; pa.t, first four prearticular teeth; pa.b, prearticular dermal bone; sy, symphyseal tooth.

events in the development of the dentition. This approach is a correlative and comparative one to achieve the most from the scarce source of *Neoceratodus* eggs. Functional studies were limited to the use of cyclopamine added to the water in which free-living, or early released embryos were individually kept. This was used to block shh signal before most of the teeth were initiated but long after the early embryonic events. Experimental fish were compared with control fish to determine the affects on tooth development, at a later stage when numbers of teeth would have developed.

The early patterning events in *Neoceratodus* show features in common with the stereotypical osteichthyan dentition, both from gene expression and morphological data. A novel observation is that the early temporal pattern of induction of tooth sites is one that we find in other osteichthyan dentitions.

2. MATERIAL AND METHODS

Embryos and larval stages of *N. forsteri* are a scarce and precious resource with eggs collected in the period September–December and individually stored, recorded and staged as in the tables (see website below). With many varied systems being studied, this necessitates restriction of genes and stages to any one study. Lungfish eggs were collected from the spawning ponds established at Macquarie University. Each egg, and after hatching each fish, was kept separately in shallow, sterilized pond water (changed every 3–4 days). After the first 2–3 weeks post hatching at stage 47, they had used up the residual yolk and begun feeding first on brine shrimp and later on bloodworms (both treated with antibiotic prior to feeding).

Website for staging of developing lungfish: <http://www.bio.mq.edu.au/dept/centres/lungfish/lungfishresearch.html>

(a) Skeletal preparations

Control and experimental fish were processed as described by Dingerkus & Uhler (1977) for visualization of cartilage and bone. These were examined with differential interference microscopy (DIC) from whole embryo stained and cleared skeletal preparations. Flat mounts were prepared as jaws dissected from the Alizarin red and Alcian blue preparations, to count all teeth, including those that had just begun to

develop as tooth tips, and to estimate developmental age to compare with tables of dentition stages at each embryological stage in Kemp (1977). Drawings were made to compile the complete, staged growth series in figure 1. Photographs were taken on either a Wild M3Z or a Zeiss Photomicroscope III with Nomarski Optics (DIC) and digitally recorded with a Nikon Coolpix 990.

(b) DNA cloning and in situ hybridization

Partial sequences of Nfshh and Nfpitx2 were amplified from embryonic total RNA by RT-PCR using degenerate primers. Using sequence-specific primers, each full-length cDNA was isolated by 5' and 3' rapid amplification of cDNA ends (RACE) using SMART RACE cDNA Amplification Kit (CLONTECH). All sequences have been deposited with the DNA Databank of Japan—AB449520 Nfshh, AB449251 Nfpitx2. Whole mount *in situ* hybridization for embryos was performed by a standard protocol for *Xenopus* embryos, as described previously (Sive et al. 2000).

(c) Cyclopamine experiments

All fish were staged and subsequently kept separately in 20 ml of sterilized pond water; those at stage 42 were kept after treatment until stage 47. Experimental fish had added to their pond water either 4 μ l of a 5 g ml⁻¹ cyclopamine (LC Laboratories in New Haven, CT, USA) in ethanol or a 2.5 g ml⁻¹ cyclopamine in ethanol. Control fish had added to their pond water 4 μ l of ethanol. Each of the three groups had their 20 ml of pond water changed at the same time daily with the appropriate cyclopamine in ethanol or just ethanol added. Half of the fish for each group were exposed to treatment for 4 days and the remaining half for 8 days. This plant-derived, steroidal alkaloid cyclopamine was used by Wada et al. (2005) to block hedgehog signalling in the zebrafish. On completion of the above regime, all the fish were maintained separately in sterile pond water until they required feeding. Since the inhibition of shh affected development of teeth and branchial cartilages, both being required for feeding, the experiment was terminated at this time, by anaesthetizing each fish in MS222, followed by fixation in neutral buffered formalin. Wada et al. (2005) noted in zebrafish that treatment of older stage embryos, comparable with the lungfish embryos, disrupted the development of the chondrocranium. They

also noted that suppression of the *shh* signal persists long after the cessation of cyclopamine addition to the water in which they were living.

3. EXPERIMENTAL OBSERVATIONS

The results for *Nfshh* show explicit sites of focused expression in the dental epithelium located in the tooth sites. Moreover, *shh* expression is intense at a later time and in a pattern different from that of *pitx2* (see the electronic supplementary material). Variation in the intensity of *shh* expression coincides with the differences in timing of each tooth germ, specifically in the marginal teeth of the lower jaw. Not only did this changing pattern of *shh* expression mark loci for the tooth initiation events in a time sequence, but expression intensity also varied at each tooth stage. These results could only be explained by comparison with the detailed order of timing at sites of tooth initiation from the skeletal preparations of Alizarin red- and Alcian blue-stained and cleared specimens. We show in skeletal whole mounts that the tooth order is from 2, to 3 and then 1 in the lower jaw marginal teeth. Often the left teeth are earlier to form than the right equivalents (figure 1*f*) and all are initiated after the symphyseal tooth. None of these marginal teeth form after larval stage-specific cyclopamine treatment.

(a) Sequence of tooth development data

The earliest teeth develop superficially in the endodermal lining to the primitive mouth (figure 1*a,b*) from interaction with cranial neural crest-derived mesenchyme (Kundrat *et al.* 2008). This occurs before the mouth is open or continuity of the ectoderm and endoderm is completed, at *Neoceratodus* stage 43 when cells of the endoderm extend as far as the lips (Kemp 2002). Epithelial–mesenchymal interactions for initial tooth development in tetrapods also occur between the same embryonic germ layers (Graveson *et al.* 1997). On the inner aspect of each tooth germ developed earlier, a cluster of cells (protogerm) forms at the level of the basal epithelium. This is the putative region for the next tooth germ to develop in the primary series of teeth (arrow, figure 1*a,b*) and is located within the dental epithelial cells around the earlier tooth. This superficial tooth development for primary teeth in *Neoceratodus* is identical to that of the rainbow trout and occurs without formation of a dental lamina first (Fraser *et al.* 2006*b*). Secondary teeth never appear, as exceptionally the primary teeth are not replaced in the dentition of any lungfish. As part of the tooth module in all vertebrates, each tooth germ forms an individual bone of attachment (figure 1*a*, arrow head; figure 1*i–k*, *ba*). This is the only bone to develop for the lower jaw marginal dentition in these lungfish, as the dermal bone for a dentary is absent, but the inner tooth plates are joined to the prearticular dermal bone (figure 1*d*). In the upper jaw, marginal teeth for the equivalent of the pre-maxilla and maxilla never develop and the inner or palatal teeth are joined to the pterygoid and vomerine dermal bones. The tooth module itself with its own bone of attachment (dental bone) develops separately from the jaw cartilages and the dermal bones (Smith & Hall 1993). The earliest stages of tooth development and older stages in the lower jaw could easily be distinguished, viewed with DIC, as first tooth tips; then larger cones and finally taller cones with dental bone could

be distinguished around the base (stages 39–52; figure 1*c–g*; figure 2*c–h*). The temporal sequence of tooth addition in the marginal (dentary) row was observed and drawings were made at each morphological stage of larval and hatchling development (figure 1*h–m*) to compare with the dentition stages published by Kemp (1977). After the first teeth on the prearticular bones appeared (figure 1*c,h*; *pa.t*, *pa1–pa4*), a single midline tooth anterior to the symphysis between the prearticular bones was the first of the marginal dentition to form (*sy*, figure 1*c,h*) and all teeth were too young to have formed the bone of attachment. Following this, marginal tooth positions were established on the Meckel's cartilage of the lower jaw from the first ones at jaw position 2, left and right pioneer teeth (*d2*; figure 1*c,i*). The next teeth develop at position 3 (*d3*; figure 1*c,i*), and then a third more distal tooth at position 1 (*d1*; figure 1*d,j*). This constitutes the triad of teeth proposed by Smith (2003) as the first to establish pattern on each dentate bone in all osteichthyans. As seen from the labial side in the next stage, one tooth (figure 1*e,k*, *d4*) is added proximally to the 'dentary' row.

Drawings of the order of tooth initiation are shown with teeth on the left side only (figure 1*i–l*), whereas (*h*) and (*m*) show both sides from the earliest recorded to the oldest. The stage drawn in (*k*) is where the bone of attachment joins the first three teeth and the fourth (*d4*) in a posterior position has been initiated, but its bone has not formed (figure 1*k*). The tooth at jaw position 1 (*d1*) does not generate an adjacent tooth bud but behind tooth 3 (*d3*), row extension continues in a posterior sequential pattern (figure 1*l,m*, *d5*, *d6*), until seven are formed. Without any secondary teeth forming, resorption of the row starts with loss of the symphyseal tooth and dentary tooth 1 (*sy*, left *d1*, figure 1*m*). This process continues until all teeth and bone have been removed and no further teeth are added. The pattern of tooth loss by resorption of each tooth in sequence is not regular, but tends to start from anterior to posterior, first 1, then 2, then 3 and continues as the last tooth is added in the row (see fig. 1*b* in Ahlberg *et al.* 2006).

An experimental disruption of tooth formation was achieved in *N. forsteri* by administration of cyclopamine at the earliest practical stage of larval development, when only three prearticular teeth had started to form. Embryos were then left to develop over a period where many more teeth would have started to form until stage 47. As seen by comparison of the control and experimental lower jaws at the same stage of development, all marginal teeth of the dentary are missing as is the symphyseal tooth (figure 1*f,g*). Initiation of all the marginal teeth had been stopped as had addition of teeth to the prearticular dentition. This is interpreted as a loss due to inhibition of *shh* signalling in the tooth differentiation pathway.

(b) In situ gene expression data

The data from *in situ* hybridization analysed from selected morphological stages (40–42, 44–45, 47) mark the timing of commitment to tooth differentiation with a differential expression of *Nfshh*. This has revealed intrinsic time order differences of each tooth for its induction and effectively refines data from the skeletal preparations. The whole mounts of *shh in situ* hybridization of upper and lower jaws showed a differential intensity at tooth loci between upper

and lower jaws (figure 2a,b), with the lower jaw showing most intense expression and an asymmetrical distribution. We find that this reflects the induction timing differences between teeth at stage 45; one vomerine (vo1) and three pterygoid (pt1–3) teeth are present on each side (figure 2c). The faint expression loci in the upper jaw could be localized to sites of new pterygoid teeth at a very early initiation stage but not to any vomerine teeth at this time (arrow heads, figure 2d–f). As observed in a skeletal preparation at an earlier time, the single vomerine teeth (figure 2c, stage 40) did not have new adjacent teeth forming, nor did the three pterygoid teeth. The two faint expression loci of one side of the jaws were positioned anterior to the three pterygoid teeth already formed (figure 2d,e) and behind (posterior) the vomerine teeth (figure 2f). Later at stage 47, in a cleared skeletal preparation with cartilage in blue, the new pterygoid teeth can be seen anterior to the older teeth (pt.t, figure 2g,h), whereas the new second vomerine teeth (vo2) are posterolateral to the first one (vo1, figure 2g,h). Similarly, the positions of these new teeth can be seen at stage 47 as calcified tooth tips without attachment bone in an Alizarin-stained preparation (vo2, pt.t, figure 2h). At this stage, upper jaw teeth were at the initial stage of induction with weak *shh* expression and contrasted with intense expression of the outer row of lower jaw teeth (figure 2a,b).

In contrast to the weak *shh* expression of the upper jaw pterygoid teeth at stage 45, marginal teeth of the lower jaw showed very strong expression of *shh* (figure 3a–h). These teeth are labial to the larger and older teeth of the prearticular bone, where new expression sites were not seen. The explanation for this differential in expression intensity is that it reveals the cryptic different time points for each tooth initiation stage in the dentition. A distinct time order was observed at larval stages 44–45 and correlated with the skeletal stages of the first three teeth formed on the lower jaw, at jaw positions d2, d3, and d1 (figure 3i,j). The low power view of *shh* expression loci (figure 3a) shows the asymmetry between left and right, as detected in the jaw whole mounts through optical sectioning (*z*-shift focus levels), with the tooth in left position 1 as strong expression and right tooth 1 as weak (l.d1, r.d1, figure 3b). On the left side of the jaw dentary teeth are initiated earlier than their equivalents on the right side (figure 3a–d). On the left side, no expression remains in tooth 3, nor in the older one at 2 (figure 3c), whereas, on the right, tooth 3 has very strong expression and tooth 1 has a weak expression (figure 3d). These teeth are compared at a higher resolution and at different optical section levels (figure 3e–h) which clearly show that those on the left side (d1, figure 3e) are in advance of those on the right (d1, figure 3f). The tooth at position 1 on the right side is beginning expression of the gene (low level, d1, figure 3f) after that of the left (high level, d1, figure 3e). The third tooth on the left has reduced expression (none, d3, figure 3g), whereas the equivalent on the right is intense (high level, d3, figure 3h). We can show that the time order correlates with the gene expression intensity, as on the right side (figure 3d,f,h) the tooth at position 3 is the strongest, 2 (oldest) is not expressing *shh*, and 1 is faint (last to form). In the skeletal preparation, size of the tooth cones equates with time since induction (figure 3i, d1–d3) and dental bone is present in the oldest tooth (d2, figure 3i,j).

4. DISCUSSION

Our results show that the gene for sonic hedgehog is reiteratively expressed at each tooth site along the jaw and is required to initiate tooth development in the lungfish. The requirement is shown by our experimental inhibition of tooth production with cyclopamine. In these experimental larval fish, kept until the later hatchling stages, all new teeth had been prevented from forming. Contemporaneously, two other studies of patterning dentitions have shown that initiation of teeth is dependent on *shh* signalling by using cyclopamine to block signalling (i) in the snake *Python sebae* (Buchtova et al. 2008) and (ii) in the cichlid *Cynotilapia afra* (Fraser et al. 2008). Most importantly the pattern of their initiation is an osteichthyan one, with previously unrecognized stereotypic sites from a pioneer, or commissioning tooth followed by adjacent teeth (Smith 2003; Fraser et al. 2004, 2006a,b; Huyssseune 2006).

Comparison of the *in situ* gene expression data with the timed skeletal stages for tooth development provides a control to identify the sequence of *shh* gene activation at each tooth site. *shh* expression is notably different from the expression pattern of *pitx2*, where the latter is probably involved in commissioning the tooth sites. The absence of *pitx2* later in development may correlate with lack of replacement teeth (see the electronic supplementary material). The timing of commitment to tooth differentiation is a short-timed phase in dental development, as evidenced by differential intensity of *shh* expression. The *in situ* hybridization with a probe for *shh* shows focused expression loci within the dental epithelium and a precise time sequence at each tooth locus, changing from faint to intense gene expression and then none. We have concentrated on the observed expression of *shh* sequentially along the lower jaw marginal teeth as these can most easily be compared with other osteichthyans. Variation in *shh* expression correlates with the dentition pattern order and is propagated in a regulated sequence with a unique time for each tooth site along the jaw, notably different times for left and right sides. As the expression time for each tooth was relatively short, it was possible to demonstrate different timings of equivalent tooth positions. We concluded that this timing of maximum *shh* expression in the tooth bud confirms observations of a different timing of tooth development for left and right in skeletal preparations. We propose that if subsequent data reveal the same pattern in the differentiation of dentitions of other species, then *shh* temporal asymmetry may be universally important and key to determining distinct left–right morphologies with mirror image polarities.

We know from Eberhart et al. (2006) that early hedgehog signalling organizes craniofacial development in the zebrafish, although they admit that the precise roles in individual steps remain largely unresolved. They concluded that the earliest function of hedgehog signalling is to regulate the development of the stomodeum (oral ectoderm or endoderm) and its subsequent interactions with post-migratory cranial neural crest cells (ectomesenchyme). As discussed, the early development of teeth in *Neoceratodus* depends on these interactions between *shh*-expressing oral epithelium and mesencephalic-derived neural crest (Kundrat et al. 2008) for odontoblasts to differentiate and make teeth.

Figure 2. *In situ* hybridized *Nfshh* whole mounts of embryos at stage 45. Comparison with upper jaw skeletal preparations, at stages 40 (c) and 47 (g,h) to show order of tooth development, stained with Alcian blue for cartilage and Alizarin red, calcified bone and dentine. (a,b) Anterior view of *shh* expression with focus on (a) the lower jaw tooth sites and (b) the upper jaw shows fainter expression in the upper tooth loci than in the lower ones. (c) Stage 40 flat slide mounted upper jaw viewed with DIC; tooth cones are transparent in ventral view above the trabecular cartilage (Alcian blue stain); two vomerine teeth of left and right (vo1) are anterior to the three larger size, right pterygoid teeth (pt1–3). (d–f) Upper jaw dissected from the embryo in (a,b), seen in DIC, shows the weak expression of *shh* within a cluster of cells which represents the tooth placode for each of four tooth buds (arrow heads), as the next to develop in the pterygoid sets antero-lateral to the earlier formed pterygoid teeth; (d) left and right sides with four tooth loci; (e) right side jaw of the field in (d) with two loci; (f) midline field from (d), shows the tiny level of expression in the tooth placode for the left pterygoid new tooth site, there is no expression and no placode lateral to the vomerine teeth. (g) Stage 47 cleared upper jaw ventral view with DIC, Alcian blue for cartilage, with clear zones for the tooth buds of vomerine (vo2) and pterygoid (pt.t) tooth plates. (h) Stage 47 cleared upper jaw with Alizarin red stained teeth (vo1, 2, pt.t); the position of the newest vomerine teeth is lateral and that of the pterygoid anterior is relative to each of the older teeth, coincident with the earlier position of the *shh* loci.

Smith (2003) postulated that each pioneer tooth site (the first in each dentate field) regulates subsequent adjacent tooth formation, so that interruption of *shh* signal during early dentition pattern stages would prevent all teeth in the series from forming. As shown here, cyclopamine given before initiation of this tooth series prevented the first symphyseal tooth from forming, and all

others in *Neoceratodus*. Although no teeth formed in the marginal dentary series, very early teeth on the prearticular bone prior to treatment were retarded in their development from the time of exposure to treatment and no additional teeth were formed. This shows that sequential adjacent teeth require a signal from the pioneer tooth to be initiated. New results on cichlid fish

Figure 3. *In situ* hybridized *Nfshh* whole mounts of lower jaw at stage 45. (a,b) Flat mount preparations dissected from specimen in figure 2 in DIC showing the localization of differential *shh* expression to tooth germs on the dentary row, compared with skeletal preparation at the same stage (i,j) to show sequence of dentary tooth order. (a) Whole field view of three dentary teeth (arrows) either side of the symphyseal tooth (sy) shows asymmetric expression loci of *shh* as observed in dorsal view and coincides with the sequential time series of tooth germs (figures 1j and 3i,j). (b) Midline field tooth 1 (l.d1) shows intense expression and is much stronger than the equivalent tooth on the right (r.d1). (c,d) At higher magnification to compare left (c) and right (d), only d1 shows strong expression on the left, whereas d1 is weak and d3 intense on the right. (e,f) High resolution of tooth germs left and right to show localization of the expression product to the cells of the tooth placodes (d1) but not the older tooth germs (sy, d2); the youngest to form d1 right is weak and d1 left is intense, with the more advanced symphyseal tooth (sy) and (d2) negative. (g,h) Compare high-resolution view of second with form (d3), left is no expression and right is intense. (i) Cleared Alcian blue and (j) drawing (see colour code in figure 1) to show time order of tooth development in dorsal view of the first three teeth to form after the symphyseal one, with d2 the oldest (bone formed), d3 younger (dentine cone only) and d1 the youngest (the smallest in (i)).

(Fraser *et al.* 2008) showed the role of the pioneer tooth as a source of signals promoting the iterative sequence of teeth, by cyclopamine inhibition of *shh*. Inhibition of tooth induction experimentally, is assumed to result from blocking *shh* signal by antagonism of the signal-activation component (Chen *et al.* 2002). Cobourne *et al.* (2004) showed in the mouse mandible that control of initiation of tooth position was dependent on *shh* signalling. We can

assume that a similar mechanism operates in *Neoceratodus* when an edentulous state is produced experimentally with cyclopamine inhibition of *shh* pathways.

The lower jaw marginal tooth series is the only part of the otherwise specialized dentition in *Neoceratodus* likely to be homologous with that of other osteichthyans. We have now found that in the dentary tooth row, the order of development starts with a symphyseal tooth, then position

2, next 3, followed by 1. We propose that this initial pattern order is stereotypic of the dentary of osteichthyans as shared with that identified in actinopterygian osteichthyans. That is, the same pattern order is shown for dentary teeth in two teleost species: (i) *Oms hh* expression in the trout *Oncorhynchus mykiss* (Fraser *et al.* 2004), and (ii) the medaka *Orizias latipes* (Debiais-Thibaud *et al.* 2007). Further, these latter authors showed that the sequential order in timing is revealed by *eve1* gene expression in the epithelial dental placodes where gene expression is the same for both marginal and pharyngeal teeth, indicative of a gene mechanism fundamental to all jawed vertebrates. The early longitudinal anatomical studies of developing dentitions in *O. mykiss* by Berkovitz (1977, 1978) first showed just how specific the tooth pattern was for each dentate bone. Also, as recorded in the trout (Fraser *et al.* 2006a), tooth buds in *N. forsteri* form superficially and in association with the outer dental epithelium of the adjacent tooth germ rather than from a dental lamina. Each one forms as an independent module in development supported only by the individual bone of attachment (Smith & Hall 1993). The dental bone of the tooth module in *Neoceratodus* links them all together and is located on the dorsal antero-lateral edge of Meckel's cartilage as the functional support of these transitory lower jaw teeth (Smith *et al.* 2002). Any dermal ossification centre for the dentary bone is absent in the extant forms of the group (Bartsch 1993).

The question posed from previous studies of dipnoan dentitions (Reisz & Smith 2001; Ahlberg *et al.* 2006) was how regulation of developmental pattern was transformed through evolution from the conventional osteichthyan pattern. The osteichthyan template for tooth order could be transformed into a dipnoan pattern through evolution of developmental mechanisms with loss of replacement tooth formation but retention of addition to the primary tooth rows. These concepts are best explained as part of the evolving disparity in early lungfish dentitions (Ahlberg *et al.* 2006) and more fully discussed in the electronic supplementary material. Apart from the marginal teeth (dentary), the specialized inner tooth plates of upper (pterygoid) and lower (prearticular) jaws in dipnoans are dentitions radically different from all other osteichthyans. However, even these fused tooth plates also begin their development from individual and separate teeth and have one pioneer tooth for each tooth plate (Kemp 1977, 1979, 2002; Smith 1985; Smith & Krupina 2001; Smith *et al.* 2002). A pioneer tooth for each of the dentate bones, with sequential initiation of teeth on the jaw, is a pattern recognized for many teleosts (Huyseune 2006). However, tooth rows in teleosts are formed first in even tooth positions, then in odd tooth positions as an alternate second series, and this appears not to be present in lungfish. Also, lungfish do not form replacement teeth for those in the primary tooth rows. We have previously shown (Smith & Krupina 2001; Smith *et al.* 2002) that ongoing successive tooth addition is a one-directional pattern in all tooth plates even in the dentary equivalent, where this is posterior (proximal). The direction of growth of the dentition for the dentary of the trout (Berkovitz 1977; Fraser *et al.* 2004; 2006a,b) is also in a posterior direction. This unique dipnoan pattern of tooth addition without replacement has been a strongly conserved developmental process for at least 350 Myr

(Reisz & Smith 2001; Smith & Krupina 2001; Smith *et al.* 2002) as discussed by comparison of *Neoceratodus* with a superbly preserved growth series of a Late Devonian lungfish.

Contrary to the concept that the dentition of *Neoceratodus* is unique (Kemp 2002) and even built from cell types different from those employed by all other osteichthyans including tetrapods, most of the early patterning events in *Neoceratodus* are shared with these groups. We can now claim that the initial and early patterning of the marginal dentition is in a teleost temporo-spatial order, one that is highly conserved and not completely different from other osteichthyans, as we have previously thought (Reisz & Smith 2001; Ahlberg *et al.* 2006). Moreover, it has recently been shown that the migration of cranial neural crest cells as mandibular, hyoid and branchial streams (Falck *et al.* 2000; Ericsson *et al.* 2008) conforms to that of all osteichthyans including dipnoans. Post-migratory crest-derived cells from this source give rise to cells for the dental papilla of all tooth positions (Kundrat *et al.* 2008), a shared tetrapod feature. In addition we have shown that there is a common developmental process as tooth bud formation is superficial, with lack of a dental lamina and new tooth sites forming from the dental epithelium of the earlier teeth as in teleosts (Smith *et al.* in press). Also, there is a requirement for *shh* by the dental epithelial cells to initiate their differentiation and activation of tooth development. This completely contradicts previous views, such as those expressed by Kemp (2002) that 'it (tooth row in position of the dentary) is not comparable to the marginal dentitions of other vertebrates'.

This study was approved by the Animal Ethics Committee of Macquarie University.

Research grants from the Royal Society London, and the Leverhulme Trust for M.M.S., and Australian Research Council grant DP0663874 for J.J. are acknowledged; we are grateful to Anthony Graham for laboratory support and advice, and Zerina Johanson for comments on an early draft.

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