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Skull Development During Anuran Metamorphosis: III. Role of Thyroid Hormone in Chondrogenesis

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ABSTRACT Metamorphosis of cranial cartilages in anuran amphibians constitutes one of the most dramatic and extensive ontogenetic transformations in vertebrates. We quantitatively examined the role of thyroid hormone (3,3',5-triiodo-L-thyronine; T_3) in mediating gross aspects of this morphological repatterning in the skull of the Oriental fire-bellied toad, *Bombina orientalis*. T_3 was administered via plastic (Elvax) micropellets in three treatment dosages (2.5, 0.25, and 0.025 μg) and one control dosage (0 μg) to tadpoles of three Gosner developmental stages —28/29, 30/31, and 32/33; tadpoles were recoved up to 8 d (treatment and control dosages) or 14 d (control dosage) later. Response of larval cartilages to exogenous T_3 was dosage dependent but not implant-stage dependent; chondrogenic tissues that participate in metamorphic transformation are competent to respond to T_3 well before they normally do. Metamorphic effects of T_3 were visible within 2 d; in most treatment groups, the normal metamorphic sequence was two-thirds complete after 8 d. While T_3 also induced precocious ossification, the normal temporal relation between bone formation and cartilage transformation was dissociated in experimental groups. Morphological integration between cartilage and bone during cranial metamorphosis is at least partly the result of each tissue responding independently to endocrine factors.

Among the most remarkable examples of vertebrate organogenesis is the suite of changes that constitute skull development during amphibian, and especially anuran, metamorphosis. In a matter of weeks—days in some species—cranial skeletal structures are extensively modified coincident with the shift from aquatic to terrestrial habitats: cartilaginous components which predominate in the larval skull are profoundly transformed; bony components which will predominate in the adult make their initial appearance. Moreover, developmental events involving both bone and cartilage occur in a precise, orchestrated temporal sequence that achieves a high degree of morphological integration both among components of each skeletal tissue as well as between cartilage and bone.

More than a half-century of experimental research has extensively documented the primary role of endocrine factors in mediating the diverse morphological and physiological changes that occur during amphibian metamorphosis (Dodd and Dodd, '76; White and Nicholl, '81). Of especial importance are thyroid hormones (TH), which, either singly or in concert with other hormones, directly mediate changes in target tissues (Atkinson, '81; Kaltenbach, '68; Kollros, '81). These studies, however, have largely neglected the role of endocrine factors in mediating metamorphic changes in the skeletal system (see references in Hanken and Hall, '88a). This inattention is especially surprising in view of both the dominant role played by hormones, particularly TH, in skeletal development, growth, and remodeling in other vertebrates (see reviews by Jowsey and Detenbeck, '69; Nijweide et al., '86; Raisz et al., '78; Reddi, '82; Reddi and Sullivan, '80; Silbermann, '83), and the renewed interest in the developmental and regulatory processes underlying metamorphic transformation in the context of the evolution and development of morphology and its constraints (Alberch, '87; Alberch and Gale, '86; Lebedkina, '85; Roth and Wake, '85; Wake, '82; Wake and Roth, '85).

A recent paper from our laboratory described the prominent role of T_3 in cranial ossification during metamorphosis in the Oriental fire-bellied toad, *Bombina orientalis* (Hanken and Hall, '88a). In the present paper, we reveal the complementary, equally prominent role of T_3 in mediating the metamorphic transformation of cranial cartilages—changes that are in many respects more dramatic and complex than those involving the osteocranium. Data constitute both description of the normal metamorphic changes in cartilaginous components in laboratory-reared specimens and quanti-

TABLE 1. Characters used to quantify metamorphic transformation of cartilage

Character	Character state[1]
Suprarostral cartilage	0—Fully developed, or slightly resorbed at margins
	1—Resorption further advanced, some matrix still stains with Alcian Blue
	2—Absent, or matrix no longer stains with Alcian Blue
Cornu trabeculae	0—Fully developed, or beginning to resorb distally
	1—Resorbing along entire length
	2—Absent, or only trace remaining proximally
Infrarostral and Meckel's cartilages	0—Distinct, freely articulating
	1—Partly fused; external margin of mandible irregular
	2—Synchondrotically united; external margin of mandible smooth
Palatoquadrate cartilage	0—Articular process extends anterior to eye, ascending process synchondrotically united with neurocranium
	1—Articular process at or posterior to anterior margin of the eye, synchondrosis between ascending process and neurocranium degenerating
	2—Main axis of palatoquadrate vertically or posteriorly displaced from articulation of otic process with otic capsule
Ceratohyal cartilage	0—Main axis of lateral process oriented at right angles to the longitudinal axis of skull
	1—Lateral process slightly elongated and reoriented; its main axis lies at an approximately 45° angle to the longitudinal axis of the skull
	2—Elongate, S-shaped; distal end recurved
Ceratobranchial cartilages	0—Fully developed, or cartilage rays beginning to resorb at margins
	1—Resorption further advanced
	2—Absent, or only base of ceratobranchials I–III remaining adjacent to hypobranchial plate

[1]0, larval; 1, transitional; 2, postmetamorphic.

tative analysis of the response of premetamorphic tadpoles to application of exogenous T_3 via plastic micropellet implants. Finally, we offer preliminary observations concerning the role of T_3 in effecting morphological integration between cranial cartilage and bone during amphibian metamorphosis—specifically, that such integration is at least partly the result of each tissue responding independently to endocrine factors.

MATERIALS AND METHODS

Specimens used

Tadpoles of *B. orientalis* were derived from several laboratory matings among wild-caught adults that are maintained as a breeding colony. (Breeding and husbandry procedures are described in Hanken and Hall, '88b.) Descriptions of skull development during natural metamorphosis were derived from specimens used in an earlier study of cranial ossification (Hanken and Hall, '84). Additional tadpoles used for hormone implants were reared inside an environmental chamber under controlled conditions of 18° ± 1°C and an alternating photoperiod of 12L:12D.

Hormone administration

Hormone pellets were implanted into tadpoles of six different Gosner ('60) developmental stages that constitute three groups: stages 28/29, 30/31, and 32/33. These stages are all within the period of premetamorphosis (sensu Etkin, '64) and precede by several stages the gross changes in the cartilaginous skeleton that are first detectable in whole-mount preparations during the subsequent period of prometamorphosis (see below). The plastic (Elvax) micropellets containing T_3 (3,3′,5-triiodo-L-thyronine) were implanted dorsally within the dermis on the right side of the head posteromedial to the eye and lateral to the braincase. (Methods for pellet preparation and implantation are described in Hanken and Hall, '88a.) Three treatment dosages of T_3 were used: 2.5, 0.25, and 0.025 μg per pellet; a fourth type of pellet, prepared identically to the others but lacking T_3, was used in controls. In choosing T_3 we relied on recent evidence that it, and not T_4 (thyroxine), has the major role in inducing metamorphosis in target tissues (Buscaglia et al., '85). Tadpoles of each treatment regime (dosage × implant stage) were recovered 2, 4, 6, and 8 d after receiving the implant and fixed and preserved in neutral-buffered formalin before histological processing; additional samples of control tadpoles were allowed to continue until 14 d post-operation.

Skeletal preparations

Before staining, preserved specimens were scored for developmental stage and measured (snout–vent

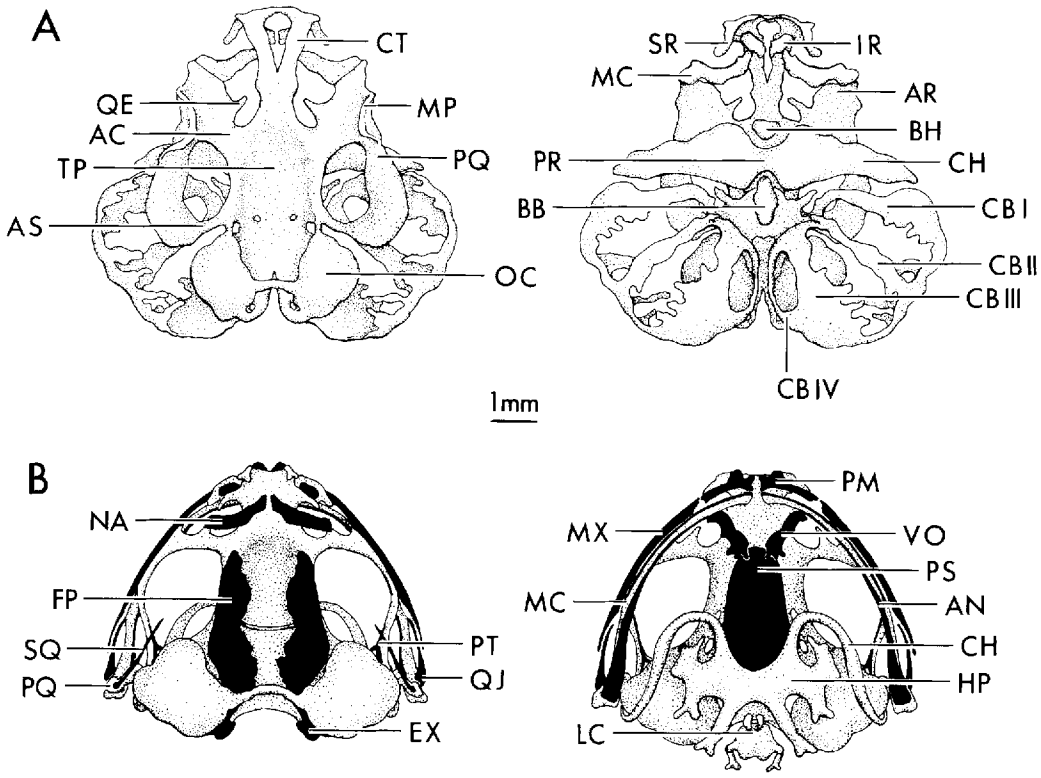

Fig. 1. Pre- and postmetamorphic skulls of *Bombina orientalis*. **A:** Larva, Gosner stage 36. **B:** Postmetamorphic froglet, stage 46. Left, dorsal views; right, ventral views. Abbreviations: AC, anterior commissure; AN, angulosplenial; AR, articulating process; AS, ascending process; BB, basibranchial cartilage; BH, basihyal cartilage; CB I–IV, ceratobranchial cartilages I–IV; CH, ceratohyal cartilage; CT, cornu trabeculae; EX, exoccipital; FP, frontoparietal; HP, hypobranchial plate; IR, infrarostral cartilage; LC, laryngeal cartilage; MC, Meckel's cartilage; MP, muscular process; MX, maxilla; NA, nasal; OC, otic capsule; PM, premaxilla; PQ, palatoquadrate cartilage; PR, pars reuniens; PS, parasphenoid; PT, pterygoid; QE, quadrato-ethmoid process; QJ, quadratojugal; SQ, squamosal; SR, suprarostral cartilage; TP, trabecular plate; VO, vomer.

length, tail length); general descriptions of external features in control and treated tadpoles are presented in Hanken and Hall ('88a). They were then prepared as cleared whole-mounts differentially stained for bone and cartilage with Alizarin Red and Alcian Blue, respectively (Hanken and Wassersug, '81; Wassersug, '76). Formation of new cartilage could be readily observed in these specimens; progressive remodeling and loss of larval structures were identified by the degeneration and poor staining of extracellular matrix that characterized cartilage resorption.

Quantification

To quantify cartilage transformation in both naturally metamorphosed and T_3-treated specimens, all specimens were scored for six different cartilage characters that change substantially during metamorphosis (Table 1). Each character could assume one of three states: 0, larval configuration; 1 transitional (i.e., midmetamorphic) configuration; 2, postmetamorphic configuration. Character-state values for all six characters were then summed to derive an overall value for each specimen—the cartilage index—that could total between 0 (premetamorphic larva) and 12 (postmetamorphic froglet).

RESULTS

Normal development of cartilage

General features

Metamorphic changes in anuran cranial cartilages have been described in detail for several species, such as *Rana temporaria* (de Jongh, '68; Pusey, '38), *Bufo regularis* (Sedra, '50; Sedra and Michael, '58), *Heleophryne purcelli* (van der Westhuizen, '61), and *Ascaphus truei* (van Eeden, '51). Notwithstanding differences in specific aspects, general features characteristic of these taxa apply to most anurans, including *Bombina* (de Beer, '37; Pusey,

'38). The following is a brief review of the six character transformations that we used to quantify cartilage metamorphosis in *B. orientalis*. Readers interested in more extensive descriptions of these characters are referred to the above papers. Static descriptions or illustrations of larval and adult skulls in *Bombina* may be found in Sokol ('75),[1] Slabbert ('45), Ramaswami ('42), van Eeden ('51), and Wassersug and Hoff ('82) and in earlier papers cited therein. Larval and adult skulls are illustrated in Figures 1 and 2.

The *cornu trabeculae* (trabecular horns) are exclusively larval components of the neurocranium. When fully developed, these paired cartilages diverge first anteriorly and then ventrally from the anterior margin of the trabecular plate. Medial processes join the cornu anteriorly, which thereby enclose a median fenestra. They are resorbed during metamorphosis, beginning at the anterior end; the remaining stumps become incorporated into the posteroventral components of nasal capsules.

The paired *suprarostral cartilages* are lateral, triradiate extensions from the anterior margins of the cornu trabeculae that form the functional upper jaw in the tadpole. They are completely resorbed at metamorphosis, but only after the cornu trabeculae have disappeared. Consequently, they pass through a stage when they appear as thin, transverse cartilages isolated from the remainder of the cranium.

The *palatoquadrate cartilage* consitutes the jaw suspension both before and after metamorphosis. In the larva it is a prominent, paired cartilage synchondrotically united with the trabecular plate of the neurocranium in two places: anteriorly, via the broad anterior commissure; posteriorly, via the ascending process which contacts he neurocranium immediately anterior to, yet separate from, the otic capsules. In addition, a quadrato-ethmoid process provides ligamentous attachment with the ipsilateral trabecular horn. At its anterior end, which extends in front of the eye when viewed in dorsal aspect, the palatoquadrate articulates with Meckel's cartilage; ventral to its broad muscular process it articulates with the ceratohyal cartilage. This configuration is drastically altered at metamorphosis. First, the cartilage shortens and rotates so that ultimately it descends ventroposteriorly from its articulation with the neurocranium to the jaw joint. Second, the anterior commissure connecting the palatoquadrate and neurocranium is resorbed. In its place, however, the palatoquadrate establishes a new connection with the neurocranium via the quadrato-ethmoid process (now termed the pterygoid process) which articulates with and then fuses to the newly formed posterior maxillary process extending posterolaterally from the nasal capsule. Third, the posterior cartilaginous connection with the neurocranium (ascending process) is also resorbed, and a new articulation is established between the otic process of the palatoquadrate, derived from the remainder of the muscular process, and the neurocranium; these latter elements may later fuse. Fourth, the articulation with Meckel's cartilage is retained through metamorphosis, albeit highly modified; the articulation with the ceratohyal is lost.

Infrarostral and *Meckel's cartilages* each are paired components of the mandibular arch that form the lower jaw in both larval and postmetamorphic stages. In the larva, they are arrayed in a transverse series: from their lateral articulation with the paired palatoquadrate cartilages, Meckel's cartilages articulate with the infrarostral cartilages, which in turn articulate medially; together, they form an expanded "M" when viewed from the anterior aspect. In addition, both cartilages are curved—the concave side facing anteriorly in Meckel's, posteriorly in infrarostral. At metamorphosis, this configuration is altered primarily in two ways. First, the functional joints between Meckel's and infrarostral cartilages and between the two infrarostral cartilages disappear. In their place, the once-articulating elements are synchondrotically (Meckel's-infrarostral) or syndesmotically (infrarostral-infrarostral) united to form a single, smoothly curved rod of mandibular cartilage. Second, Meckel's cartilage elongates tremendously at its posterior end such that the articulation with the palatoquadrate cartilage migrates posteriorly, coincident with the reorientation of the jaw suspension.

The *ceratohyals* are paired, triradiate cartilages which, together with the median, larval basihyal cartilage, are components of the second, or hyoid, arch. In the larva, the transverse ceratohyals are joined to one another, and to posterior ceratobranchial and basibranchial cartilages, via the cartilaginous pars reuniens; dorsolaterally, each ceratohyal articulates with the adjacent palatoquadrate cartilage. At metamorphosis, they undergo extensive reorientation and remodeling. By a combination of proliferation and resorption, lateral

[1]Figure 8 of Sokol ('75) depicts a "mature" larval skull of *B. orientalis*, reconstructed from serial sections, that has a third connection between the palatoquadrate cartilage and the neurocranium via a larval otic process. None of the more than 100 cleared and stained larval specimens that we have examined has this process. Sokol's description thus would appear to be incorrect in this respect.

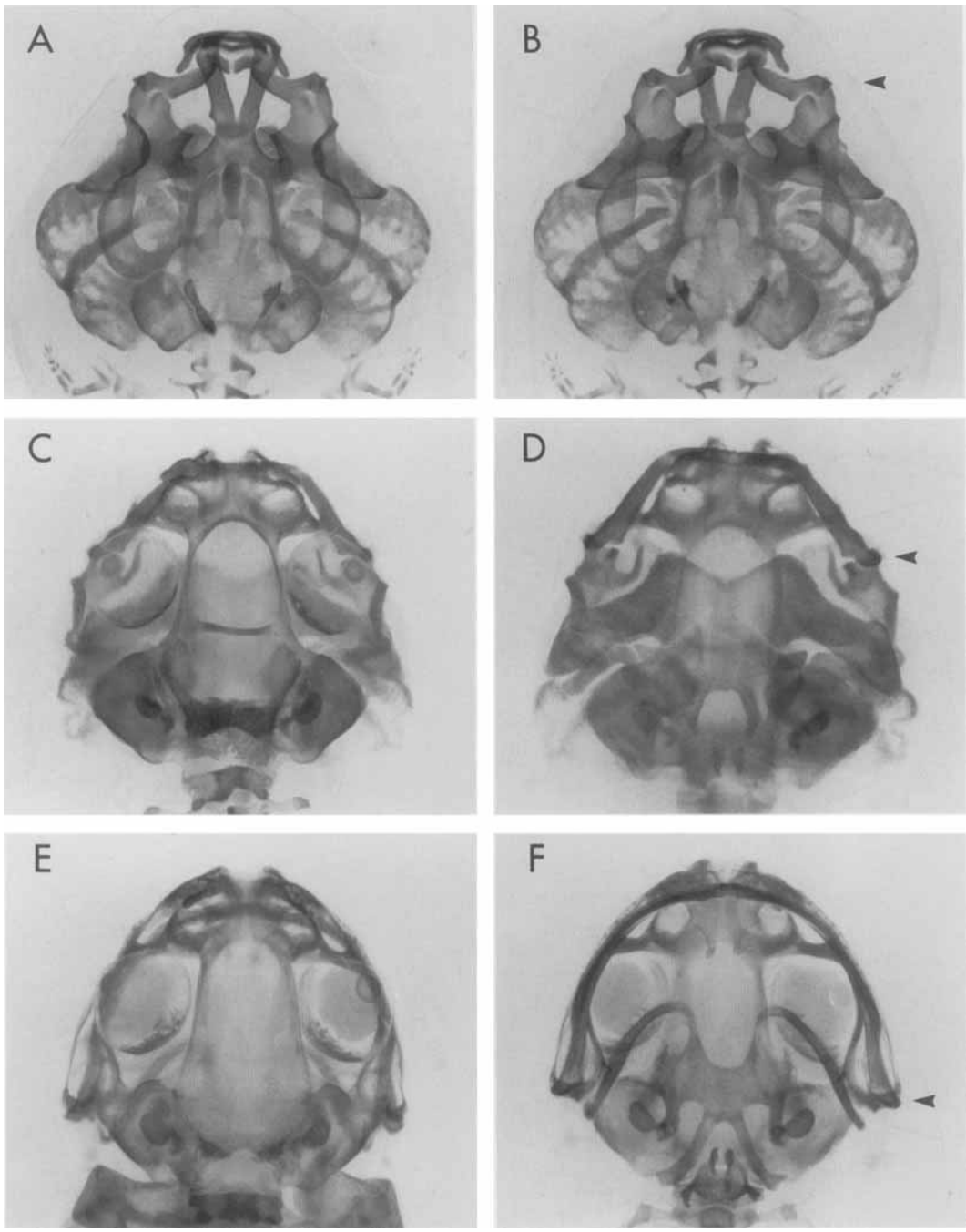

Fig. 2. Photomicrographs of (A,B) larval (Gosner stage 36), (C,D) midmetamorphic (stage 43), and (E,F) postmetamorphic (stage 46) skulls of *B. orientalis*. A,C, E—dorsal views; B,D,F—ventral views. Progressive changes in each of the six cartilage characters (described in text and identified in Fig. 1) are clearly visible. Note, for example, resorption of cornu trabeculae and suprarostral cartilages by stage 43, resorption of ceratobranchial cartilages by stage 46, and posterior migration of jaw articulation between palatoquadrate and Meckel's cartilages (arrowheads).

processes elongate and are reshaped while the distal tips are drawn posteriorly, accompanying the posterior migration of the palatoquadrate cartilages with which they still articulate. Simultaneously, median regions proliferate to form the anterior portions of the broad hypobranchial plate. Finally, each ceratoyal loses its articulation with the adjacent palatoquadrate cartilage, establishing a new, syndesmotic junction with the neurocranium.

Four paired *ceratobranchial cartilages* and associated basal elements compose the larval gill basket. At metamorphosis, all four cartilages are resorbed, beginning at their dorsal margins. In addition, the hypobranchial plate expands and is remodeled, incorporating the anterior basibranchial, median portions of the ceratohyals, and possibly the base of one or more ceratobranchials. Laryngeal cartilages also form from de novo condensations at this time.

Quantitative description

Many aspects of metamorphosis of different cartilages proceed simultaneously. Nevertheless, differences among cartilages in the relative timing of natural metamorphic transformation are apparent when mean scores for each character are plotted against developmental stage (Fig. 3, Table 2). Among neurocranial derivatives, cornu trabeculae both begin (mean score > 0) and complete (mean score = 2) metamorphosis an average of one stage earlier than do the suprarostral cartilages. Among first-arch elements, changes in Meckel's and infraorbital cartilages proceed in advance of those in the palatoquadrate cartilage by approximately two stages. Progressive changes, however, involving the two hyobranchial components—ceratohyal and ceratobranchial cartilages—are virtually contemporaneous.

Values of cartilage index, constituting the sum of individual character-state scores for each specimen, reveal the rapid rate of cartilage transformation during natural metamorphosis (Fig. 4). Transformation is first detected quantitatively (mean cartilage index > 0) at stage 41, and it proceeds at an approximately steady rate, relative to Gosner developmental stage, beyond this point. A "perfect" transitional state (mean index = 6) is attained between stages 42 and 43, and metamorphosis is complete (index = 12) in all specimens within a few days of attaining stage 46 upon the completion of metamorphosis.

The abruptness of cartilage transformation during metamorphosis stands in sharp distinction to the more protracted sequence of appearance of cranial ossification centers (Fig. 4). Onset of osteogenesis precedes by several Gosner stages the earliest phases of cartilage transformation; three bones—the frontoparietal, the parasphenoid, and the exoccipital—are visible in all whole-mount preparations as early as stage 38, and in fact have differentiated and are visible in serial sections as early as stage 30 (Hanken and Hall, '84, '88a). Similarly, only 13 of the adult complement of 17 skull bones are visible immediately upon completion of metamorphosis (stage 46), whereas the gross aspects of cartilage transformation are virtually complete at this time; the remaining bones will not form until several months later (Hanken and Hall, '84; Hanken, unpublished data).

When considering these data it is important to remember that representing each cartilage character by only three states obscures the gradual nature of each transition and precludes description of subtle aspects of timing. For example, the larval state (score = 0) for each character includes the earliest stages of metamorphic transformation which commonly occur one Gosner developmental stage before the specimens attain the transitional state (score = 1). Quantification of metamorphic transformation as used in this study is for two primary purposes: depicting differences in the relative timing of the metamorphosis among cartilages during normal development and evaluating the response of larval cartilages to exogenous hormone administration. It does not depict the absolute timing of initiation or completion of metamorphic transformation.

Cartilage development following T_3 treatment

General features

Exogenous T_3 initiated the full sequence of metamorphic transformation that characterizes normal development. Depending on the dosage and the treatment interval, the effects ranged from slight to extensive (Table 3). Indeed, in all respects but one the nature and sequence of changes were typi-

TABLE 2. Cartilage transformation during natural metamorphosis[1]

Gosner stage	Mean cartilage index	S.E.	Range
36	0	0	—
—			
38	0	0	—
—			
40	0	0	—
41	1.0	0.45	0–2
42	4.6	0.60	4–7
43	8.2	0.37	7–9
44	9.6	0.24	9–10
45	11.4	0.60	9–12
46	12.0	0	—

[1]Cartilage index equals the sum of all six character-state scores in each specimen. Stage 46 specimens were preserved within 1 month of completing metamorphosis. N equals 5 specimens per stage.

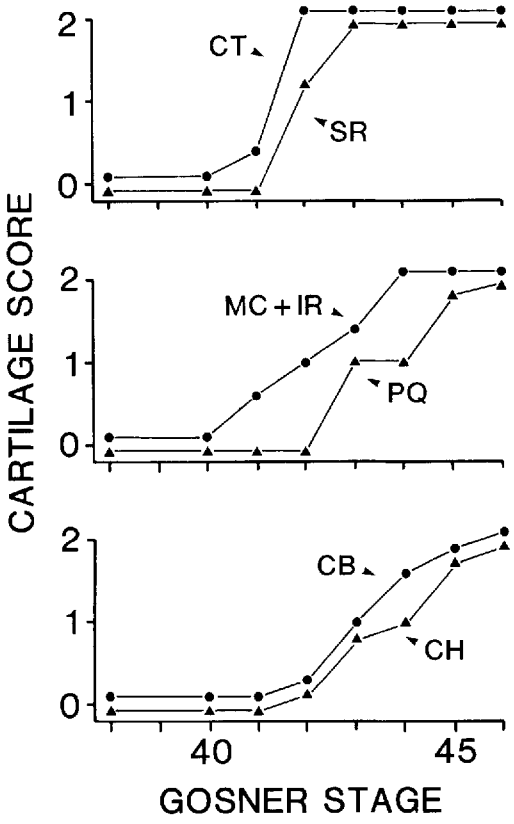

Fig. 3. Timing of transformation of individual cartilage characters during natural metamorphosis. **Upper graph:** neurocranial components—cornu trabeculae (CT) and suprarostral cartilages (SR); **middle graph:** first-arch splanchnocranial components—palatoquadrate cartilages (PQ), and Meckel's and infrarostral cartilages (MC + IR); **lower graph:** hyoid and posterior arch splanchnocranial components—ceratohyal (CH) and ceratobranchial cartilages I–IV (CB). For each cartilage character, character-state scores range from 0 (larval configuration) to 2 (postmetamorphic configuration); N = 5 for each value.

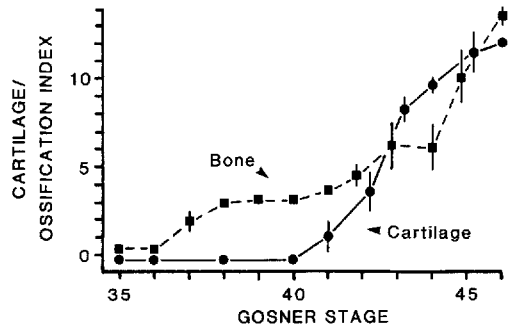

Fig. 4. Timing of overall cartilage transformation and bone formation during natural metamorphosis. Cartilage index (●) equals the mean sum of all six individual character state scores for each specimen; N = 5 for each value. Ossification index (■) equals the mean number of bones visible in the same whole-mount specimens (paired bones count as one-half point for each side; maximum equals 17); N = 10 for all stages except 46 (N = 3). Vertical bar, ± 2 S.E. Ossification data are from Hanken and Hall ('84).

cal of those seen during natural metamorphosis. The sole exception was the rate of change: at all T_3 dosages, transformation began earlier than in controls and proceeded at a faster pace than during normal metamorphosis (Table 3; Hanken, unpublished data). In many treatment groups, especially at higher dosages, specimens showed a positive response to T_3 as early as 2 d after pellet implantation (Table 3, Fig. 5). All 11 such cases showed partial resorption of the cornu trabeculae; two of these specimens in addition showed partial resorption of the suprarostral cartilage. Despite the fact that the hormone pellet was implanted unilaterally in all specimens, there was no differential response between right and left sides; cartilage transformation always was symmetrical. Predictably, cranial skeletal metamorphosis was reflected in conspicuous changes in the external shape of the head and in the relative positions of the mouth, external nares, and eyes (Fig. 6; described in Hanken and Hall, '88a).

Quantitative description

Response to hormone administration was clearly dosage dependent (Table 3, Figs. 7, 8). Among controls, only one of 84 specimens recovered up to 8 d after pellet implantation showed any sign of cartilage metamorphosis; this specimen, implanted at stage 30/31 and recovered after 8 d, had suprarostral cartilages in transition (score = 1). Indeed, even 14 d after implantation, 20 of 21 control specimens were still unchanged; the single exception, which was implanted at stage 32/33, had both cornu trabeculae and infrarostral and Meckel's cartilages in transition (cartilage index = 2). At the lowest T_3 dosage, 0.025 μg T_3, mean cartilage index values after 8 d were higher than in controls for two of the three implant stages—28/29 and 30/31—but these differences were not statistically significant ($P > .05$); the third implant stage—32/33—gave no response (mean index = 0). At higher dosages, however, cartilage index values after 8 d were significantly higher than in both control and low-dosage groups, regardless of implant stage ($P \leq .05$); predictably values were highest at the highest dosage, 2.5 μg T_3.

The highest cartilage index value observed in any treatment group was 9, attained by four spec-

TABLE 3. Cartilage transformation following T_3 treatment[1]

Dosage (μg T_3)	Implant stage	Days after implant 2	4	6	8	14
Control	28/29	—	—	—	—	—
	30/31	—	—	—	0.1 ± 0.14	—
	32/33	—	—	—	—	0.3 ± 0.29
0.025	28/29	—	—	0.9 ± 0.40	0.8 ± 0.40[2]	
	30/31	—	0.3 ± 0.18	1.1 ± 0.59	0.7 ± 0.33[2]	
	32/33	—	0.1 ± 0.14	0.2 ± 0.17[2]	—	
0.25	28/29	0.3 ± 0.18	3.1 ± 0.14	4.9 ± 0.46	5.4 ± 0.37	
	30/31	0.3 ± 0.18	3.0 ± 0.38	5.6 ± 0.30	6.3 ± 0.18	
	32/33	—[2]	3.0 ± 0.26[2]	4.4 ± 0.57	5.4 ± 0.48	
2.5	28/29	0.3 ± 0.21[2]	4.0 ± 0.44	6.3 ± 0.29	8.0 ± 0.44	
	30/31	1.0 ± 0.31	3.6 ± 0.61	5.6 ± 0.20	7.4 ± 0.53	
	32/33	—	3.9 ± 0.51	5.3 ± 0.42	8.1 ± 0.40[3]	

[1]Cartilage index (± S.E.) equals the sum of all six individual character-state scores for each specimen. N = 7 specimens for each score except as indicated.
[2]N = 6 specimens.
[3]N = 8 specimens.

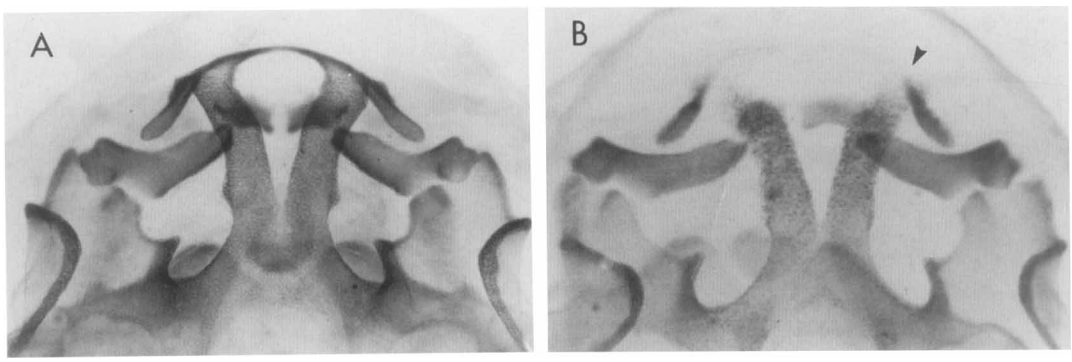

Fig. 5. Anterodorsal portions of (A) control and (B) T_3-treated skulls after 2 d. Resorption of cartilaginous cornu trabeculae and suprarostral cartilages is already underway in B (arrowhead); this tadpole received 2.5 μg T_3. The control specimen is unchanged. Both specimens received implants at stage 30/31.

imens implanted with the highest dosage at stage 32/33 and recovered after 8 d (Table 3). In all of these specimens, metamorphosis was complete or nearly complete for four characters—suprarostral cartilage, cornu trabecula, infrarostral and Meckel's cartilages, and ceratobranchial cartilages; in each of them, the palatoquadrate cartilage was in transition (score = 1). In three of the specimens, however, the ceratohyal cartilage was only slightly modified from its larval configuration (score = 0); in the fourth specimen, it was in transition. During normal metamorphosis, a mean cartilage index score of 9 would not be expected until approximately stage 43, which would not be reached until 2–3 weeks after stage 33 at the rearing temperature used in our study.

In contrast to the obvious dosage-dependent response, cartilage metamorphosis in response to T_3 was virtually stage independent. That is, for a given T_3 dosage and treatment interval, nearly all treatment groups responded to the same extent, regardless of implant stage (Table 3, Figs. 9, 10). For example, among both low-dosage and high-dosage groups, as well as among control groups, cartilage index values after 8 d are not statistically different ($P > .05$). Indeed, in only one of the 12 possible pairwise comparisons among implant stages of the same treatment dosage are the mean cartilage index values after 8 d significantly different. This case entails a comparison between specimens implanted with the intermediate dosage at stages 28/29 and 30/31, in which specimens im-

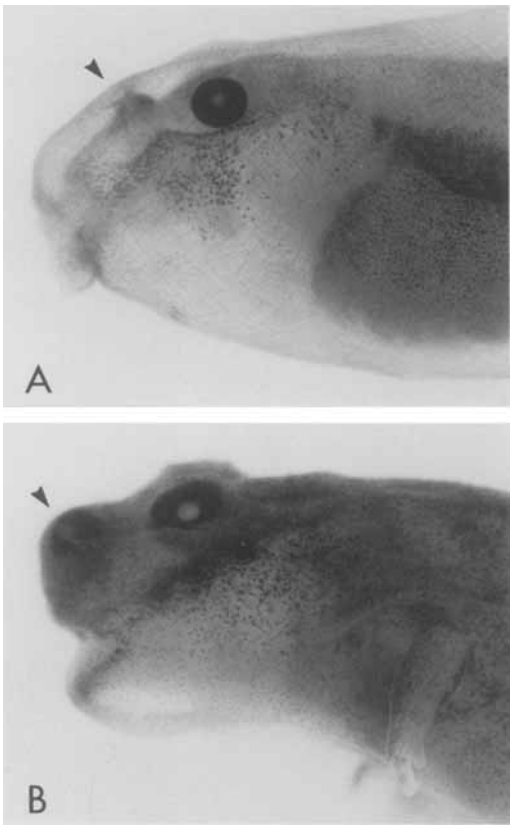

Fig. 6. External cranial morphology in (A) control and (B) T_3-treated tadpoles after 8 d. The control specimen is virtually unchanged from the time of implant. Extensive modifications visible in the treated specimen, which received 2.5 μg T_3, include the bulging eye and regression of circumorbital connective tissues, the more prominent nostril (arrowheads), and widening of the mouth and loss of horny beaks. Note also the protruding forelimb in B. Both specimens received micropellets at stage 30/31; in each, the implant site lay immediately posterior to the right eye.

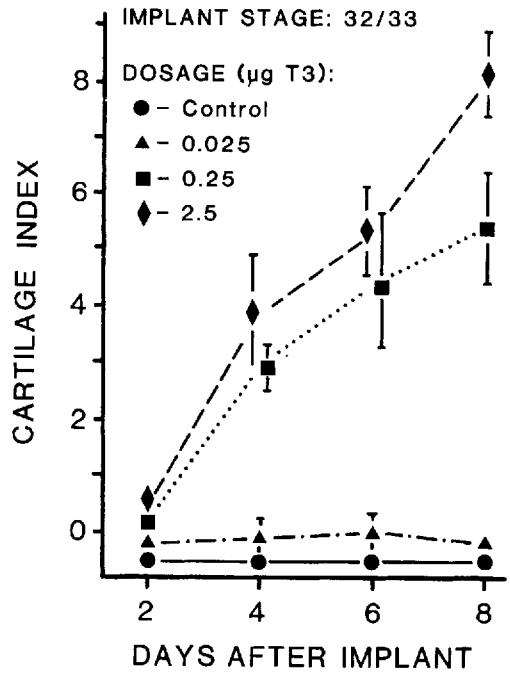

Fig. 7. Dosage-dependent response of cranial cartilages to exogenous T_3. Dosage dependence was characteristic of all implant stages (Table 3); these specimens received pellets at stage 32/33. Vertical bar, $\pm$ 2 S.E.

planted at the later stages had a cartilage index value approximately 1 point higher than that of specimens implanted at the lower stages. The index value for the oldest implant stage, 32/33, was not significantly different from that of either earlier implant stage.

The relative timing of cartilage transformation and ossification was very different in T_3-treated groups. Unlike the situation in natural metamorphosis, in which gross aspects of transformation always follow the appearance of several bones which are clearly visible in cleared and stained preparations (Hanken and Hall, '84, '88a, '88b), bone was visible in very few of the T_3-treated specimens, including those in which cartilage transformation was far advanced (Figs. 8, 10). Consequently, the profiles of cartilage and bone development during natural metamorphosis are inverted during T_3 treatment, at least with respect to the early stages of ossification (ossification index $\leqslant$ 3) which were initiated by hormone administration (cf. Figs. 4, 11).

DISCUSSION

Cartilage transformation during anuran metamorphosis entails profound changes of cranial morphology. In effect, virtually the entire cranium is structurally repatterned as the larval skull, well suited for feeding and respiration in an aquatic medium, is converted to a skull adapted for terrestrial existence. These changes have been examined in sometimes exhaustive detail for nearly a century; numerous descriptions of cranial skeletal metamorphosis in anurans and urodeles have been marshaled as evidence both for and against various theories of tetrapod origins and relationships and of the basic organization and structure of the vertebrate head (e.g., van Eeden, '51; Pusey, '38). The primary subject of our study, however, lies in a different direction: the role of endocrine factors, particularly T_3, in mediating the observed changes.

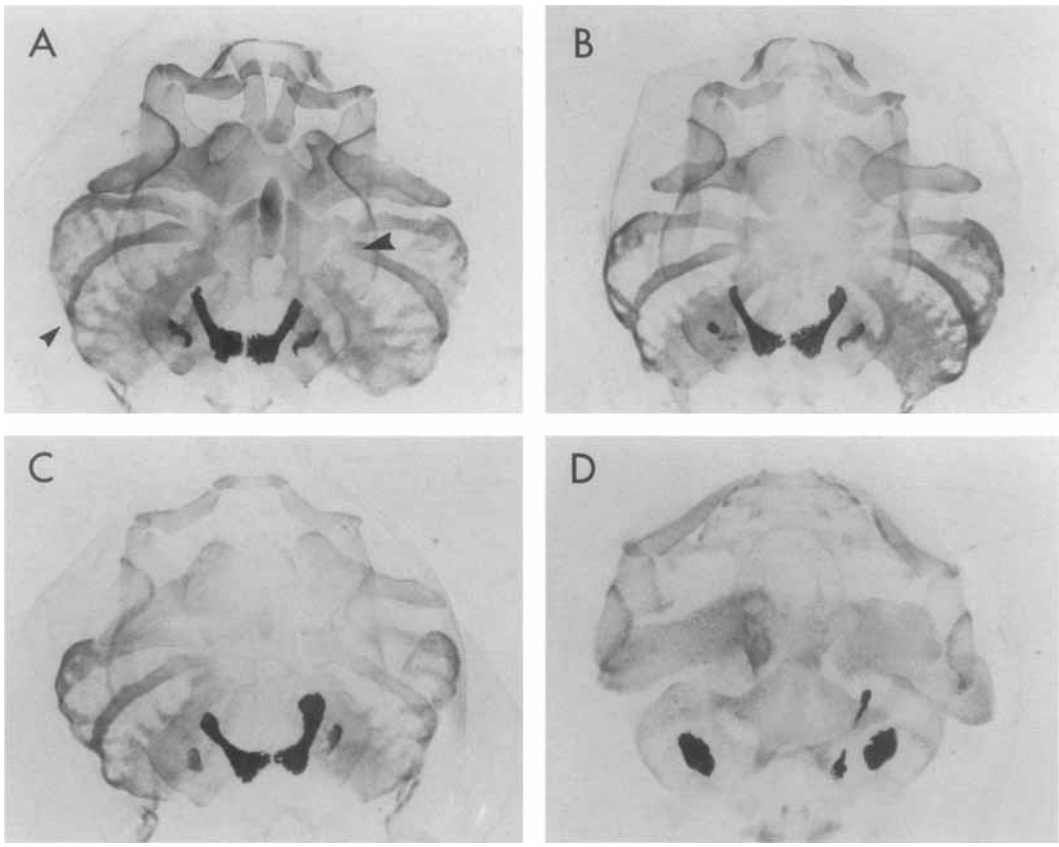

Fig. 8. Dosage-dependent response of cranial cartilages to exogenous T_3. Specimens received the following dosages of T_3: **A**, 0 (control); **B**, 0.025; **C**, 0.25; **D**, 2.5 μg. All specimens received implants at stage 32/33 and recovered after 8 d; intact transparent pellet is visible in A (large arrowhead). Note progessive transformation of cartilages with increasing dosage. For example, the ceratobranchial cartilages are present in control and lower treatment dosages (small arrowhead in A) but are fully resorbed at the highest dosage. Even at the highest dosage, however, no bone is visible in these whole-mount preparations.

Perhaps the most unexpected result in our study is the lack of a stage-dependent response of larval cartilages to hormone administration during the premetamorphic period. Stage dependence is a characteristic feature of hormone-mediated skeletal development (Burch and Lebovitz, '82a; Fell and Mellanby, '55, '56; Turley et al., '85; Takakubo et al., '86; Thomas and Nathanielsz, '83) and is only one example of the more general phenomenon of critical periods during growth and development (Scott, '86). Indeed, stage-dependent response to exogenous T_3, reflecting the timing of differentiation of ossification centers, was a conspicuous feature of an earlier study of endocrine mediation of cranial ossification in the same specimens (Hanken and Hall, '88a). In the present study, however, all stages responded equally to each dosage of T_3 by initiating cartilage metamorphosis well in advance of controls. Clearly, chondrogenic tissues that participate in metamorphic transformation are competent to respond to T_3 well before they normally do.

How, then, are absolute timing and temporal integration of cartilage metamorphosis effected during normal development? We suggest that the answer lies in differential sensitivity of individual cartilaginous tissues to T_3—specifically, that events early in metamorphosis are triggered by a lower concentration of T_3 than are later events. Other endocrines or paracrines involved in chondrogenesis, such as insulinlike growth factors I and II (IGF) and growth hormones (Ellis et al., '81; Froesch et al., '76; Isaksson et al., '82; Schoenle et al., '82), and in metamorphosis of other tissues, such as prolactin and corticosteroids (Clemons and Nicoll, '77; Frieden and Naile, '55; Kikuyama et al., '82),

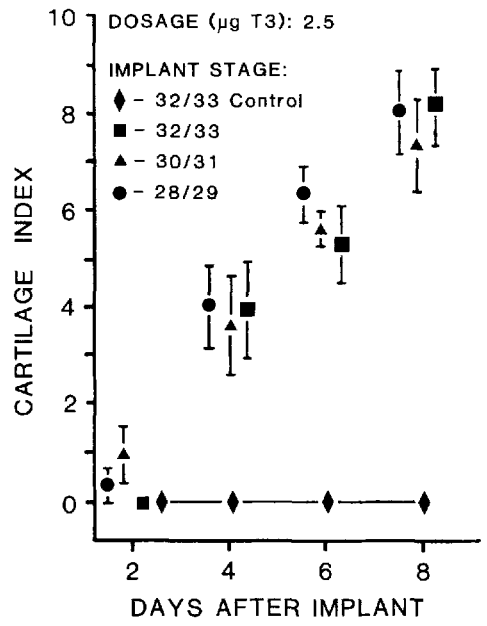

Fig. 9. Stage-independent response of cranial cartilages to exogenous T_3; implant dosage equals 2.5 μg. Cartilages remained essentially unchanged in all control specimens, regardless of implant stage; only controls for stage 32/33 are depicted here. Vertical bar, ± 2 S.E.

may similarly mediate the timing of cartilage transformation through differential sensitivity of chondrogenic tissues to these factors or by affecting tissue sensitivity to other factors, especially T_3 (Blatt et al., '69; Froesch et al., '76; Suzuki and Kikuyama, '83). Such a model, based on TH, has been proposed to explain the stereotyped series of changes that characterizes metamorphosis of the nervous system (Kollros, '81), the tail (Chou and Kollros, '74), and the osteocranium (Hanken and Hall, '84, '88a) in anurans. Furthermore, this model is supported by data obtained in this study which document an obvious dosage-dependent response over the range of T_3 amounts used: in virtually all comparisons among specimens recovered after a given treatment interval, metamorphosis was furthest advanced in the group receiving the highest dosage of T_3.

These data, however, must be interpreted with caution. Because all T_3 groups were maintained for a relatively short period (8 d at most), we are not able to distinguish the differential-sensitivity hypothesis from an alternate hypothesis that dosage primarily affects only the *rate* of metamorphosis, rather than its ultimate extent. In other words, the extent of cartilage metamorphosis in low-dosage specimens might ultimately have equaled that in high-dosage groups, had we allowed them to survive longer. Our in vivo experiment, while supporting the differential-sensitivity hypothesis, is not an unequivocal test. Such evidence must come from future analyses, particularly in vitro studies.

Mechanism of hormone action

A recurring question in the study of the role of TH in skeletal growth and development concerns the site and specificity of hormone action: are hormonal effects mediated by direct action of TH on the target tissue, or are the effects mediated indirectly via some intermediate chemical messenger whose synthesis or release is regulated by TH? Numerous studies of amphibians, using either implants in vivo or organ culture in vitro, support the idea of direct action (Kaltenbach, '53a–c; Kuhn and Hammer, '56; Niki et al., '82; additional references in Kaltenbach, '85). Additional support comes from analogous studies of amniotes (Adams and Jowsey, '67; Burch and Lebovitz, '82a,b; Fell and Mellanby, '55, '56; Jowsey and Detenbeck, '69). While the originally simple model of direct action has had to be modified by recent complicating discoveries, such as the apparent role of T_3 in enhancing the activity of IGFs (somatomedins) in promoting cartilage growth within the growth plate (Burch and Van Wyk, '87), this model is now widely accepted.

We applied T_3 via micropellet implant in the hope that we would induce local metamorphosis of skeletal tissues in the immediate vicinity of the pellet. Such an effect on several disparate tissues, including the appendicular skeleton, was earlier obtained by Kaltenbach ('53a–c), who implanted T_3-cholesterol pellets in the leopard frog *Rana pipiens*. Although in our study the effects of exogenous T_3 on skeletal development were, in general, extremely conspicuous, no specimen showed a heightened response in the vicinity of the implant or any other asymmetry in either cartilage or bone development that we could interpret as a local effect of the pellet; exogenous T_3 initiated a systemic effect. In view, however, of the convincing evidence in favor of a direct effect of TH on skeletal tissues generally, we interpret this result as simply indicating dispersion of the exogenous hormone throughout the head, either by diffusion across cranial tissues (the head is less than 7.5 mm wide in a typical stage 31 tadpole) or via the blood-vascular or lymphatic systems (capillaries adjacent to the implant site were routinely severed during surgery). Precocious metamorphosis of other, non-cranial tissues, including the tail, provides further

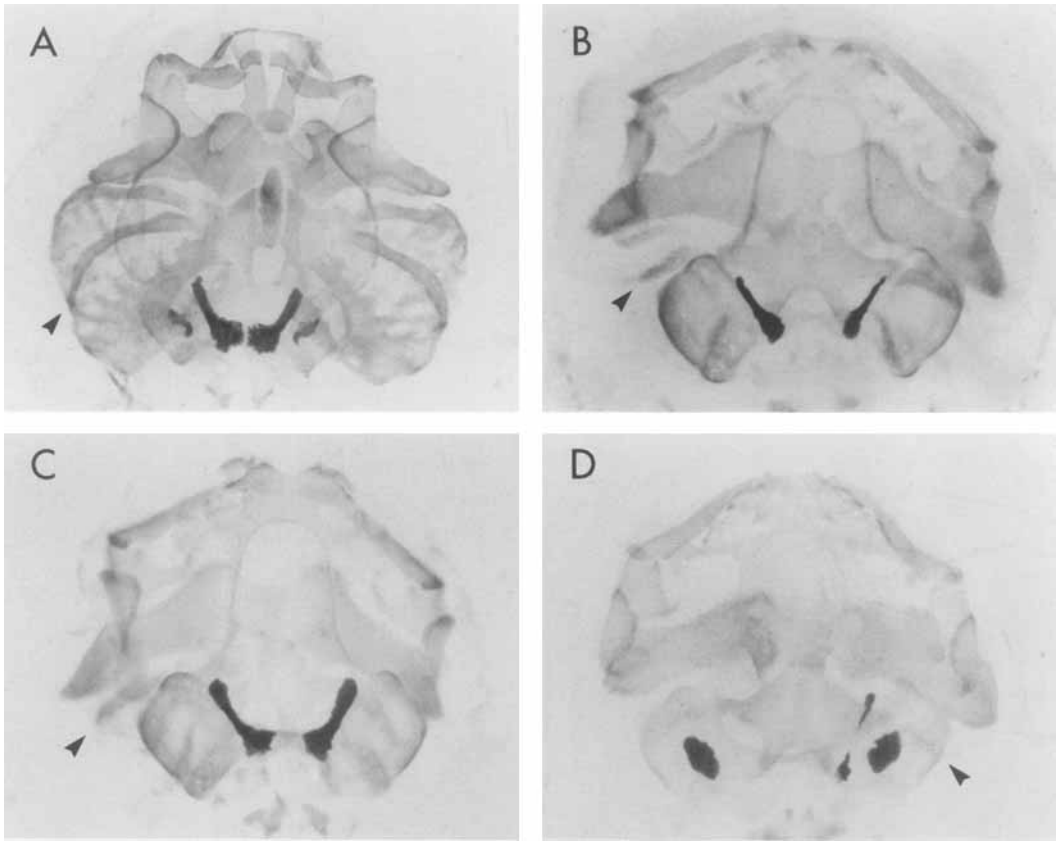

Fig. 10. Stage-independent response of cranial cartilages to exogenous T_3. **A**: Control specimen; implant stage 32/33. **B–D**: T_3-treated specimens (2.5 μg); implant stages 28/29, 30/31, and 32/33, respectively. All specimens were recovered after 8 d. All T_3-treated specimens responded to approximately the same extent; the control specimen was unchanged. Note, for example, the ceratobranchial cartilages (arrowheads) which are present in A but almost fully resorbed in B–D. No bone is visible in any specimen.

evidence of such a systemic effect (Hanken and Hall '88a). In other words, despite the absence of a local response, we interpret our results as being consistent with the idea of direct action of T_3 on the target tissue.

The scope of hormone effects

In amniotes, examination of the role of TH in regulating cartilage development has been limited largely to a rather restricted set of phenomena, viz., the growth and maturation of cartilage prior to endochondral ossification during embryogenesis or at sexual maturation (e.g., Burch and Lebovitz, '82a,b; Burch and Van Wyk, '87; Fell and Mellanby, '55, '56; Silberberg and Silberberg, '38). Based on our results, we suggest that, at least in amphibians, TH has a much more extensive role in cartilage development. Basic developmental processes that underlie metamorphic changes in the cartilaginous skeleton include complete resorption of exclusively larval structures, remodeling of additional larval cartilages via both cell proliferation and resorption, de novo condensation and differentiation of additional chondrogenic centers, loss of articulations and functional joints via chondrogenic fusion, and formation of new articulations and joints. To the extent that exogenous T_3 initiates the normal sequence of cartilage metamorphosis, all of these processes are mediated, directly or indirectly, by the hormone. Similar effects have been revealed for other hormones, such as the role of testosterone in promoting cell division of laryngeal chondrocytes and perichondrial cells in male juvenile *Xenopus laevis* (Sassoon and Kelly, '86; Sassoon et al., '86). Further detailed examination of cellular responses of a wide range of cranial cartilages to these and other hormones is needed to gain a more complete understanding of the role of endocrine factors in cranial skeletogenesis.

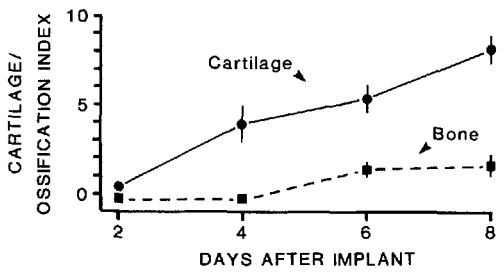

Fig. 11. Timing of overall cartilage transformation (cartilage index, ●) and bone formation (ossification index, ■) during T_3 treatment. Dosage, 2.5 μg T_3; implant stage, 32/33. The temporal relation between cartilage transformation and bone formation following T_3 treatment is the inverse of that during early stages of natural metamorphosis (cf. Fig. 4). At lower treatment dosages, in which specimens virtually lack bone entirely, the difference is even more conspicuous. Vertical bar, ± 2 S.E. Ossification data are from Hanken and Hall ('88a).

Endocrine mediation of morphological integration

Cranial skeletal metamorphosis in amphibians is an outstanding example of morphological integration during development. Two distinct tissues differentiate or transform in precise spatial and temporal patterns, effecting in a brief period transition between two complex, yet functional, end states. Are these changes in bone and cartilage developmentally linked?

In this study of cartilage and an earlier one of bone (Hanken and Hall, '88a), we document that metamorphic development of each tissue is mediated by T_3. Yet, the two tissues do not respond equally to the same treatment; cartilage responds much more quickly and to a greater extent. Consequently, at a comparable stage of cartilage metamorphosis, many fewer bones are visible in T_3-treated specimens than in normal specimens, thus inverting the normal relation between the two tissues (Figs. 4, 11). That chondrogenic tissues generally are more responsive than osteogenic tissues is, in itself, to be expected; during normal development, most of the metamorphic changes in cartilage are initiated before most skull bones appear, paralleling the pronounced increase in levels of circulating TH typical for anurans. Chondrogenic tissues thus would seem to be more sensitive to TH than osteogenic tissues. What is surprising, however, and remains to be explained, is why larval cartilages respond to exogenous T_3 before the few bones whose formation they follow during normal development. Nevertheless, three features of endocrine mediation of cranial metamorphosis clearly emerge from our results. First, T_3 treatment dissociates changes in bone from those in cartilage. Second, many significant metamorphic changes involving the two primary skeletal tissues are not tightly linked developmentally. Third, morphological integration between cartilage and bone during cranial metamorphosis is at least partly the result of each tissue responding independently to endocrine factors.

These features have important implications concerning the evolution of skull diversity. For example, evolutionary change in the timing of ossification of a particular bone associated with a change in threshold sensitivity to TH would be more likely if the bone were developmentally independent of adjacent cartilages than if they were developmentally linked. Indeed, variation in ossification timing during metamorphosis emerges as a common theme from interspecific comparisons among anurans (Trueb, '85), which seem to be more conservative with respect to the sequence of cartilage metamorphosis (Pusey, '38). In other words, independent endocrine mediation of chondrogenic and osteogenic events during ontogeny facilitates dissociation of bone and cartilage during phylogeny.

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