

Differentiation and growth of kype skeletal tissues in anadromous male Atlantic Salmon (*Salmo salar*)

P.-ECKHARD WITTEN^{*,1} and BRIAN K. HALL²

¹Institute of Marine Research at the University of Kiel, Germany and ²Department of Biology, Dalhousie University, Halifax NS, Canada

ABSTRACT The re-initiation of bone development in adult starving Atlantic salmon (*Salmo salar*) during their energetically expensive upstream migration is remarkable and deserves closer examination. Dramatic alterations of the skull bones and teeth, most prominently, the development of a kype in males, are widely known but little studied or understood. We describe the microstructure and the cellular processes involved in the formation of the skeletal tissues of the kype. Fresh bone material, obtained from animals migrating upstream was subjected to radiological, histological or histochemical analysis. We show that the kype is, in part, composed of rapidly growing skeletal needles arising at the tip of the dentary. Proximally, the needles anastomose into a spongiosa-like meshwork which retains connective tissue inside bone marrow spaces. Ventrally, the needles blend into Sharpey fiber bone. Skeletal needles and Sharpey fiber bone can be distinguished from the compact bone of the dentary by radiography. Rapid formation of the skeleton of the kype is demonstrated by the presence of numerous osteoblasts, a broad distal osteoid zone, and the appearance of proteoglycans at the growth zone. The mode of bone formation in anadromous males can be described as 'making bone as fast as possible and with as little material as possible'. Unlike the normal compact bone of the dentary, the new skeletal tissue contains chondrocytes and cartilaginous extracellular matrix. Formation of the skeleton of the kype resembles antler development in deer (a form of regeneration), or hyperostotic bone formation in other teleost fishes, rather than periosteal bone growth. The type of bone formation may be understandable in the light of the animals' starvation and the energetic costs of upstream migration. However, the structured and regulated mode of bone formation suggests that the skeleton of the kype has functional relevance and is not a by-product of hormonal alterations or change of habitat.

KEY WORDS: *Grilse, bone growth, spawning migration, kype, Sharpey fiber bone, chondroid bone*

Introduction

Alterations of the bones of the skull and the teeth occur in both sexes of Atlantic salmon (*Salmo salar*) during migration upstream to spawn; morphological changes were described in detail by Tchernavin (1937, 1938a, 1944) almost two-thirds of a century ago, and more recently by Kacem *et al.* (1998). Although all bones of the salmon skull change during migration, and although feeding teeth are replaced by a new set of teeth known as breeding teeth, the formation of a kype (hook) on the tip of the lower jaw in male salmon is the most prominent alteration (Fig. 1)¹.

The kype was the topic of scientific interest long before Tchernavin drew attention to the changes in the salmon skull. Darwin (1877) was aware that male salmon developed a kype during the breeding season. In his classic treatment, *British and Irish Salmonidae*, Day (1887) noted that "the cause of the existence of a knob on the lower jaw of male salmon and trout has been a fruitful cause of discussion from the early ages down to the present day" (p. 57).

Despite the fact that the Atlantic salmon is one of the best-studied teleost species, little information about the function of the kype is available. Hutchings and Myers (1987) suggested that male salmon use their kype to compete with other anadromous males

¹ The origins of the term "kype" are obscure. It could derive from "kip" for a hook (1615) or for a point, peak or tip (1815). A "kipper" is anything with a beak. Alternatively, it could come from "kypho" for curved, as in kyphosis for excessive rounding of the shoulders. The term "kyped," meaning hooked, can be traced to Scotland (1848), while a "kipper" is a male salmon in spawning season (Oxford English Dictionary, 2nd ed., 1989), perhaps derived from the fact that "a kip-nosed man, in Scotch, means a man with a turned-up pug nose" (Day, 1887, p. 96).

Abbreviations used in this paper: ISW salmon, grilse or salmon that have spent one winter at sea before returning to spawn for the first time; BICP/NBT, 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium; HBQ-staining, Hall-Brunt Quadruple Stain; IgG, Immunoglobulin G; K, condition factor; PBS, Phosphate buffered saline solution; TRAP, Tartrate resistant acid phosphatase; TRIS, 2-Amino-2-hydroxymethyl-1,3-propanediol.

*Address correspondence to: Dr. P. Eckhard Witten. Institute of Marine Research at the University of Kiel, Düsterober Weg 20, 24105 Kiel, Germany.
Fax: +49 (0)404-2838-3937. e-mail: pewitten@aol.com

(i.e., males swimming up river from the sea to spawn) and to fight "sneaker" males; see Table 1 for definitions of the terms for different life stages of Atlantic salmon. Järvi (1990) concluded from his experiments that the kype functions as an attractant to females at the spawning grounds. Other consequences of formation of a kype for the animals' biology — impacts on feeding behavior, repetitive spawning success, survival in fresh water, and the influence of the kype on the survival of marine stages — are even less understood (Fleming, 1996). From cellular, developmental and physiological perspectives, two interrelated facts associated with formation of the kype are remarkable: initiation of *de novo* osteogenesis in adults and formation of new bone in a life phase when the animals starve, suffer from a lack of bone minerals, mobilize their energy reserves for gonad development, undertake a metabolically expensive upstream migration, and compete with other males for females at the spawning grounds (Hutchings and Myers, 1987; Fleming, 1996; Persson *et al.*, 1998; Witten and Hall, 2001).

The present study profits from, and builds upon, the understanding of the fish skeleton contained in the fundamental work by Moss (1961a,b). Since then, considerable progress has been made, resulting in a view of fish bone which is no longer determined from the bias of studies on the mammalian skeleton. Today, similarities and differences between fish bone and mammalian bone are clearly defined (Huyseune, 2000). Furthermore, in Osteichthyes, cartilage, bone, and teeth are not regarded as separated entities, while the occurrence of intermediate tissues, such as chondroid bone, results in an enormous variety of skeletal tissues (Hall, 1975, 1978; Benjamin, 1986, 1988a,b, 1989, 1990; Huyseune and Verraes, 1990; Smith and Hall, 1990; Benjamin and Ralphs, 1991; Hall and Miyake, 2000; Huyseune, 2000). Now, fish bone is recognized as a dynamic tissue, displaying resorption and remodeling for growth and for metabolic purposes (Vielma and Lall, 1998; Persson *et al.*, 1998; Witten *et al.*, 2001). The cellular processes involved in fish bone turnover are manifold when compared to similar processes in mammalian bone (Teitelbaum, 2000). They include bone resorption by mononucleated cells, multinucleated osteoclasts, and by osteocytes, and demineralization of bone matrix (Lopez *et al.*, 1976; Hughes *et al.*, 1994a,b; Witten *et al.*, 1999a; Huyseune, 2000; Kacem *et al.*, 2000). Recent studies also demonstrate the metabolic activity of the salmon skeleton, but whether this activation mainly reflects activity in the endoskeleton or in the dermoskeleton (the scales) remains unclear (Persson *et al.*, 1998; Kacem *et al.*, 2000). Despite indications that bone metabolism in salmon migrating upstream is triggered by the needs for calcium (Persson *et al.*, 1999), there is evidence that salmon bone metabolism is basically linked to the animals phosphorous demands (Vielma and Lall, 1998; Vielma *et al.*, 1999).

Apart from a single reference to histology by Day (1887; see discussion), repeated by Tchernavin, (1944, p. 228), no information about microstructure or histology of the kype is available. Since analysis of the structure and microstructure of bone and bone cells provides information about functional and metabolic properties of the skeleton (Smith-Vaniz *et al.*, 1995), we studied the microstructural and underlying cellular processes involved in formation of the skeletal tissues of the kype. By providing basic data about the structure, growth, and dynamics of the kype, we seek both a better understanding of this unique skeletal structure and a basis for understanding its function. The present study profited, both from

access to animals from one of the world's largest and best-studied populations of Atlantic salmon — that in the Miramichi River system in New Brunswick, Canada (Chaput, 1997; Chaput *et al.*, 1998, 2000) — and from facilities which, for the first time, allowed processing of fresh tissue samples for various types of analysis.

Results

Gross Morphology

All 1SW male salmon (grilse or salmon that have spend one winter at sea before returning to spawn for the first time; see Table 1 for terminology) had a well-developed kype at the distal extremity of the lower jaw as an extension of the dentary bone (Fig. 1). Proximally, the kype is equipped with teeth but the teeth were partly covered by the oral epithelium when the fish were caught. Gross anatomical examination revealed that development of the kype involved morphological changes at the distal extremity of the lower jaw. The dentary is prolonged and curved dorsally. At the base of the kype, the height of the jaw increases (Fig. 2). No hard tissue is present at the tip of the kype, which is entirely composed of soft connective tissue (Fig. 2). When the mouth is closed, the kype fits into a deep cavity, located between the premaxillae. In grilse, the insertion of the kype into the upper jaw enables males to close their mouth, despite the presence of the kype (Fig. 2) Multi-sea winter salmon cannot close their mouths properly due to additional jaw alterations. Multi-sea winter salmon have been collected for a subsequent study.

Radiology

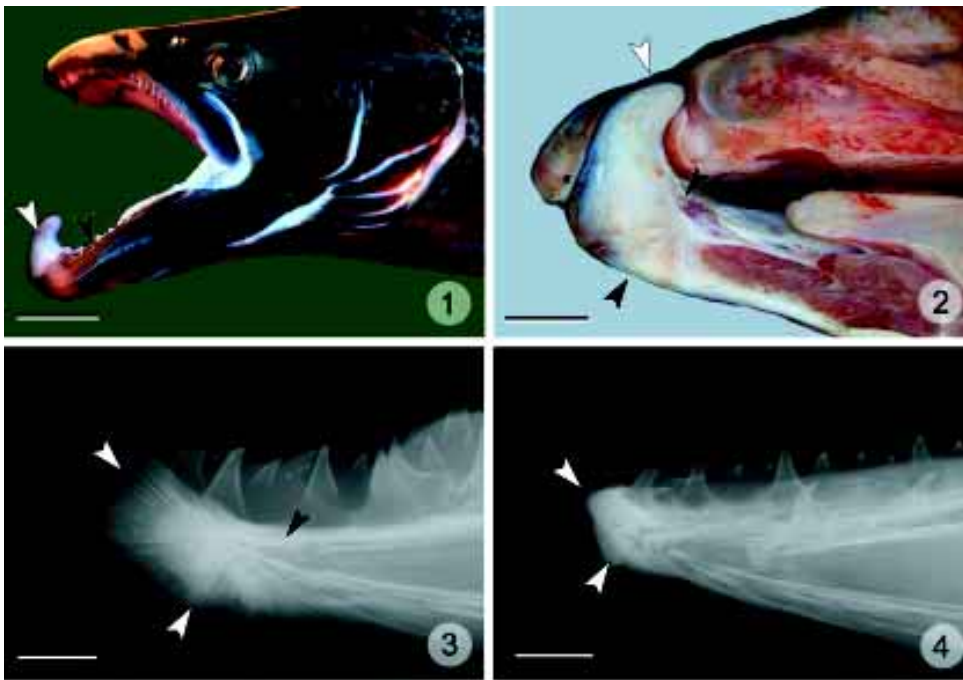
X-ray analysis of the dentary revealed that the hard tissue of the kype is composed distally of long skeletal needles that protrude distally and apically into the connective tissue of the kype (Fig. 3).

TABLE 1

DEFINITIONS OF TERMS USED TO DESCRIBE THE SALMON LIFE CYCLE

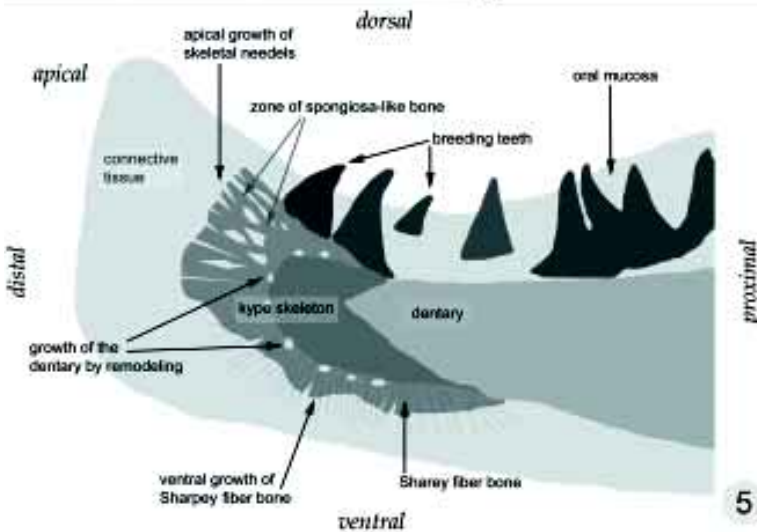
Terms	Definitions
Alevin	Yolk sac larvae
Fry	First feeding larvae after the yolk sac has been resorbed
Parr	Juvenile salmon that stay for one to four years in freshwater
Mature Parr = Sneaker males	Males that mature as juveniles and participate in the spawning of adult anadromous animals. Sneakers can stay lifelong in freshwater and become "landlocked salmon" or they can re-enter the "regular life cycle" and become anadromous salmon. Mature parr are also called precocious parr or jacks (Pacific salmon).
Smolts	Juvenile salmon, transferring from freshwater to saltwater
Landlocks	Males and females that do not migrate to the sea and become mature in freshwater
Grilse	Animals that return to their home rivers for spawning for the first time, after spending one winter in the sea (1SW = <u>one sea winter</u> salmon). Grilse usually have a fork length below 63 cm.
Salmon	Animals that return to their home rivers for spawning for the first time after spending more than one winter at sea (MSW = <u>multi sea winter</u> salmon). Also animals that have survived the first spawning and that return to their home rivers for repeated spawning, after spending one or more "winters" (years) in the sea. Salmon entering fresh water also called "bright salmon". The fork length of salmon is usually above 63 cm.
Kelts	Animals that have survived spawning. Kelts stay over winter in the river and return to the sea in the spring. Kelts are also called "black salmon".

In *Salmo salar*, animals from one population can enter different life cycles, dependent upon the age of maturation, duration of the fresh water and marine life phases, and the number of spawning events during the lifetime. To describe the different stages, terms are applied which are not commonly used in fish biology (Mills, 1989, Shear, 1992, Fleming, 1996). These terms, such as grilse and salmon, are defined above. A kype develops in male grilse and in male salmon. See Moore *et al.* (1995) for characteristics of the life cycle of animals from the Miramichie River system.



Shorter skeletal needles that extend in a ventral caudal direction broaden both the base of the kype and extend proximally into the distal part of the dentary. Skeletal needles are distinguishable from the compact bone of the dentary (Fig. 3). X-rays also show the presence of unerupted teeth associated with the new hard tissue.

Structures described so far occur in all males. Individuals differ only slightly concerning the detailed shape of the skeleton of the kype. Also, the arrangement of the breeding teeth on the hard tissue of the kype differs between individuals; the nature of the hard tissue is described below. Analysis of such variation will form part of a future study. The extent of skeletal alterations that develop in the jaws of males can be seen by comparing the male (Fig. 3) with the jaw of a female (Fig. 4) which only displays signs of rudimentary skeletal needles distally and ventrally but not apically



Microstructure

A schematic overview of the tissues of the kype and their distribution is shown in Fig. 5. Sagittal sections throughout the tip of the lower jaw confirm the presence of the skeletal needles, seen on X-rays. The needles protrude distally into the connective tissue of the kype and anastomose proximally to form a spongiosa-like meshwork (Figs. 5,6,7). Thick skeletal needles, developing on the ventral side of the jaw, merge into a solid mass of hard tissue (Figs. 6,8). As seen in X-rays, a border between the skeleton of the kype and the compact bone of the dentary is readily identifiable on the basis of organization of the bone structure (Figs. 5,6).

Skeletal Tissue and Cells

The histological features of the skeleton of the kype and of the compact bone of the dentary are listed in Table 2; see also Fig. 5 for a schematic overview. At the tissue/cellular level the skeletal needles in both distal and ventral regions of the kype display features of rapidly growing bone. Growth is indicated by the presence of prominent osteoblasts (revealed by staining with Masson's trichrome, Fig. 9), secretion of considerable osteoid (revealed by Masson's trichrome, and osteoid staining; Figs. 9,10), delayed mineralization of osteoid (revealed by Masson's trichrome and osteoid staining; Fig. 10) and the presence

Fig. 1. Head of an anadromous male grilse from the Miramichi River. The animal displays a prominent kype (white arrowhead). Breeding teeth (black arrowhead) partly protrude and are partly unerupted and covered by oral mucosa. Scale bar, 3 cm.

Fig. 2. A medial section through the forehead of a male grilse. The distal end of the dentary broadens through the development of the kype (between the two black arrowheads). The kype itself fits into a deep cavity that develops between the two premaxillae and protrudes almost through the dorsal side (white arrowhead). Scale bar, 0.75 cm.

Fig. 3. X-ray of the distal one-third of the lower jaw of a typical male grilse, showing the basic shape of the hard tissue of the kype. Variations among individuals are minor. The compact bone of the dentary (black arrowhead) is clearly distinguishable from the skeletal tissue of the kype that develops ventrally and at the tip of the lower jaw (white arrowheads). The ventral hard tissue extends into and broadens the distal part of the dentary (also see Fig. 2). The new hard tissue has a needle-like structure. Breeding teeth partly insert into the skeletal tissues of the kype and are partly covered by the oral epithelium. Scale bar, 0.4 cm.

Fig. 4. X-ray of the distal one-third of the lower jaw of a female grilse to show the differences in the basic shape of the hard tissue between the sexes and to demonstrate the extent of skeletal alterations that develop in males. Females display only a rudimentary development of skeletal needles distally and ventrally on the jaws but not apically (white arrowheads). Scale bar, 0.4 cm.

Fig. 5. Diagram of the kype of a male grilse indicating the location of the different types of hard tissues that comprise the skeleton of the kype, and providing orientation for the terms apical, distal, proximal, ventral and dorsal used in the text when referring to development of the kype and its tissues. The skeleton of the kype (dark gray) encases the tip of the dentary bone (gray) like a cap.

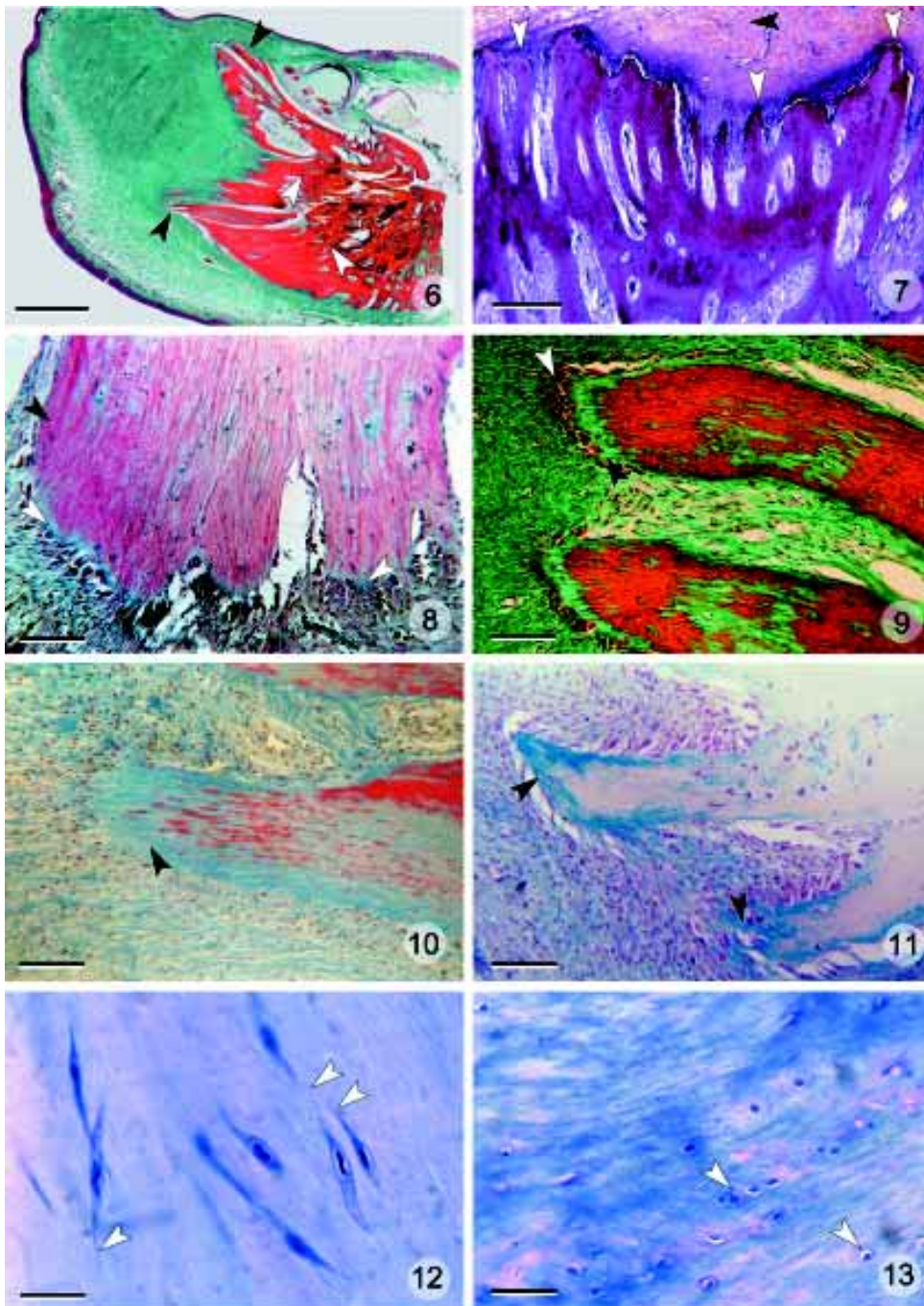


Fig. 6. A sagittal section through the tip of the dentary. As already revealed by gross morphological examination, the tip of the kype is entirely made from connective tissue (green). Needle like bone structures (black arrowheads), seen on X-rays (Figs. 3,4) arise from the periosteum of the lower jaw. The white arrowheads point to the border between skeleton of the kype and cortical bone of the lower jaw. Masson's trichrome staining. Scale bar, 0.2 cm.

Fig. 7. The boundary between the bone and connective tissue of the kype (distal above). Needle-like bone (white arrowheads) protrude distally into the connective tissue (black arrowhead) and anastomose proximally into a spongiosa-like meshwork. Toluidine Blue staining. Scale bar, 250 μ m.

Fig. 8. A view of the ventral portion of the skeletal tissue of the kype to show developing solid bone (black arrowhead). The bone formation front is rich in proteoglycans (blue staining, white arrowheads). Blue spots inside the bone matrix represent chondrocytes surrounded by cartilage matrix (see also Fig. 13). HBQ staining. Scale bar, 250 μ m.

Fig. 9. A view of the distal growth front of the kype bone (growth is from right to left) to show prominent osteoblasts (white arrowhead) at the tip of rapidly growing skeletal needles, made of cancellous bone (see also Figs. 3,4,6,7). The staining indicates a broad band of non-mineralized bone matrix (osteoid; black arrowhead) underneath the osteoblast layer. Masson's trichrome staining. Scale bar, 150 μ m.

Fig. 10. Osteoid staining reveals the presence of a broad osteoid zone (blue staining, black arrowhead) at the tip of growing skeletal needles, indicating rapid bone growth. Similar orientation to Fig. 9. Osteoid Staining. Scale bar, 150 μ m.

Fig. 11. Prominent expression of proteoglycans at the tip of a growing skeletal needle (blue staining, black arrowheads) indicates rapid growth of cancellous bone. Alcian Blue/Haematoxylin staining. Scale bar, 150 μ m.

Fig. 12. Part of the matrix of the skeleton of the kype contains osteocytes with characteristic osteocytic cytoplasmic processes (canaliculi; white arrowheads). Toluidine Blue staining. Scale bar, 15 μ m.

Fig. 13. The matrix of the skeleton of the kype also contains chondrocytes (white arrowheads), readily distinguishable from the osteocytes seen in Fig. 12 by their rounded shape, lack of cell processes, and the arrangement in cell groups. Toluidine Blue staining. Scale bar, 60 μ m.

of proteoglycans in the zone of osteoid formation (revealed by HBQ, Toluidine Blue and AH staining; Fig. 11).

Rapid bone formation, however, does not lead to the development of typical periosteal bone. Distally, a modified form of intramembranous bone formation results in the development of a chondroid bone-like tissue as bone forming cells develop into osteocytes (surrounded by bone matrix and equipped with characteristic cell processes) and chondrocytes (Figs. 12,13, and see

Table 2 for the features used to identify cells as chondrocytes). Basically the skeletal needles contain a mixture of bone and cartilage cells but locally, bone cells or cartilage cells can predominate. Hyaline cartilage is present at the distal tip of the lower jaw, joining the left and right dentary bones (Fig. 14).

Toluidine blue-, Alcian blue/Haematoxylin-, and HBQ-staining methods provide evidence for the dual presence of osteocytes and chondrocytes within the bone matrix (Figs. 8,11,12,13). In addition,

Fig. 14. Solid cartilage, both unmineralized (black arrowheads) and mineralized (red) appears distally and medially at the joint between the dentary bones in the left and right lower jaws. Masson's trichrome staining. Scale bar, 60 μ m.

Fig. 15. Treatment of sections with chondroitinase and hyaluronidase selectively removes chondroitin-6-sulphate and hyaluronic acid respectively. (Left) Non-enzyme-treated section stained with Alcian blue/Haematoxylin staining shows proteoglycans surrounding cartilage cells inside the bone matrix. (black arrowheads). (Right) Treatment with chondroitinase before staining removes chondroitin-6-sulphate from the bone (black arrowheads). Results after Hyaluronidase treatment (not shown) are similar. Scale bar, 150 μ m.

Fig. 16. Immunohistochemical demonstration of collagen type II in cartilage cells, surrounded by bone matrix. The application of a monoclonal antibody directed against collagen type II indicates that in addition to the cartilage matrix components hyaluronic acid and chondroitin-6-sulphate, cartilage cells and the surrounding matrix (black arrowheads) contain collagen type II (black arrowheads). Small osteocyte-like cells do not display collagen type II (white arrowheads). Application of a monoclonal anti-collagen type II antibody. Scale bar, 25 μ m.

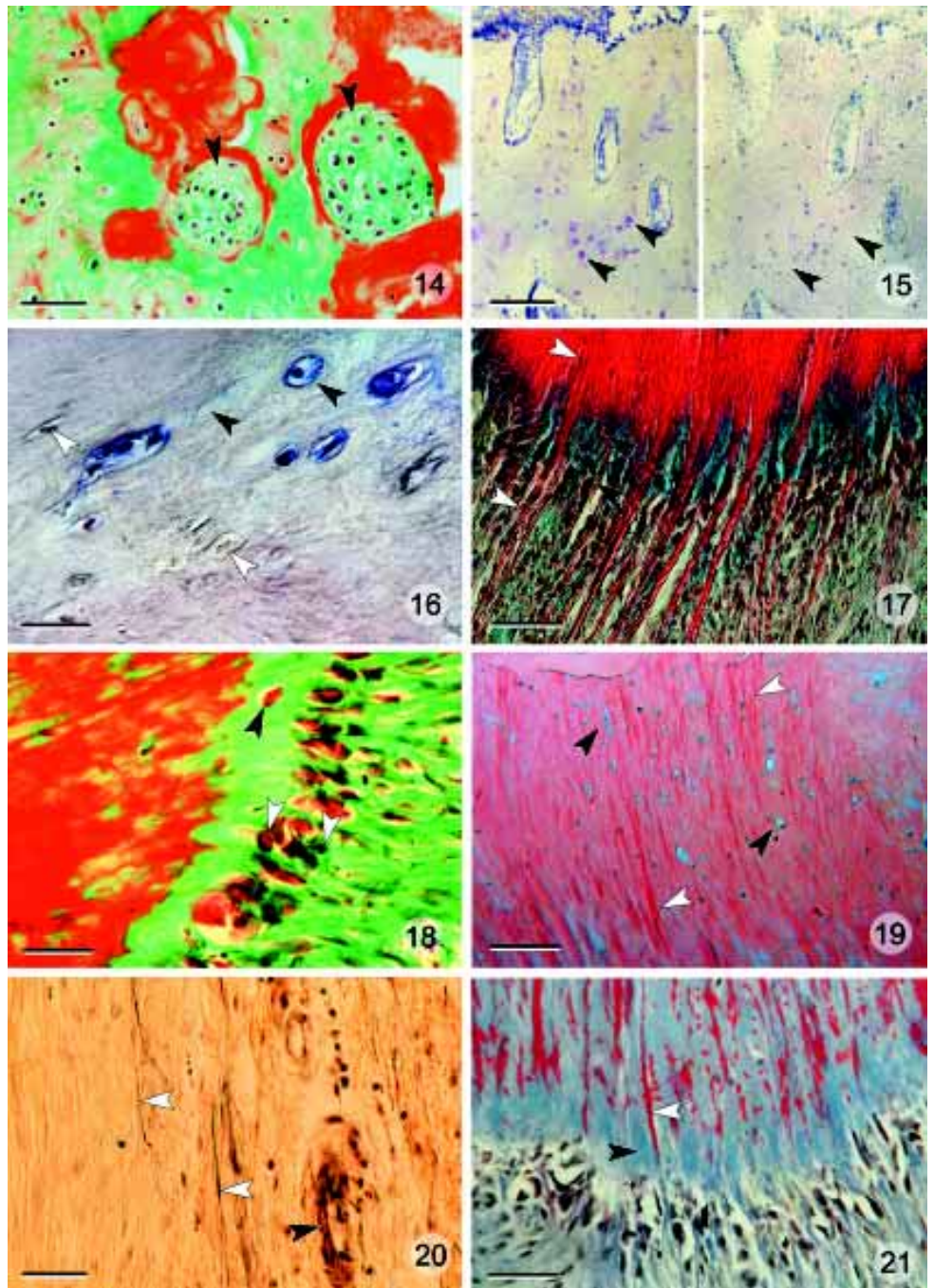
Fig. 17. Collagen fibers (white arrowheads) continue inside the matrix of the skeleton of the kype (Sharpey fiber bone) at the ventral side of the dentary. Osteoblasts arranged in multiple layers (black arrowheads), deposit new bone matrix in the gaps between the fibers. HBQ staining. Scale bar, 40 μ m.

Fig. 18. Growth of Sharpey fiber bone. As in distal bone formation in the kype (Fig. 9), the growth of Sharpey fiber bone involves the activity of prominent osteoblasts (white arrowheads). Osteoblasts secrete a broad layer of non-mineralized bone matrix that contains entrapped osteoblasts (black arrowhead). Masson's Trichrome staining. Scale bar, 25 μ m.

Fig. 19. The hard tissue of the kype on the ventral side of the dentary is Sharpey fiber bone with enclosed collagen fibers (white arrowheads). This bone also displays chondroid bone-like features, as evidenced by the enclosed cartilage cells (black arrowheads). HBQ staining. Scale bar, 100 μ m.

Fig. 20. Elastin staining indicates the presence of elastin fibers (white arrowheads) in the connective tissue of the kype. Walls of blood vessels stain also positive (black arrowhead). No elastin is detectable in the bone matrix. Verhoeff's Elastin staining. Scale bar, 200 μ m.

Fig. 21. Sharpey fiber bone. Staining that differentiates between osteoid (black arrowhead) and mineralized bone indicates that mineralization starts at collagen fibers (white arrowhead). Osteoid staining. Scale bar, 40 μ m.



the presence of cartilage-specific extracellular matrix in the bone was verified by chondroitinase and hyaluronidase digestion, which specifically remove cartilaginous ground substances, in this case, in the vicinity of cells identified on histological and histochemical criteria as chondrocytes. Enzymatic digestion results in negative proteoglycan staining with Toluidine Blue and Alcian Blue/Haematoxylin (Fig. 15).

Chondrocytes inside the bone matrix could also be labeled with a monoclonal antibody directed against collagen type II, which is the typical collagen type of cartilage (Beresford, 1993). Bone cells did not react with the antibody (Fig. 17).

The periosteal hard tissue that develops ventrally in the kype is typical Sharpey fiber bone, i.e., a bony tissue with thick collag-

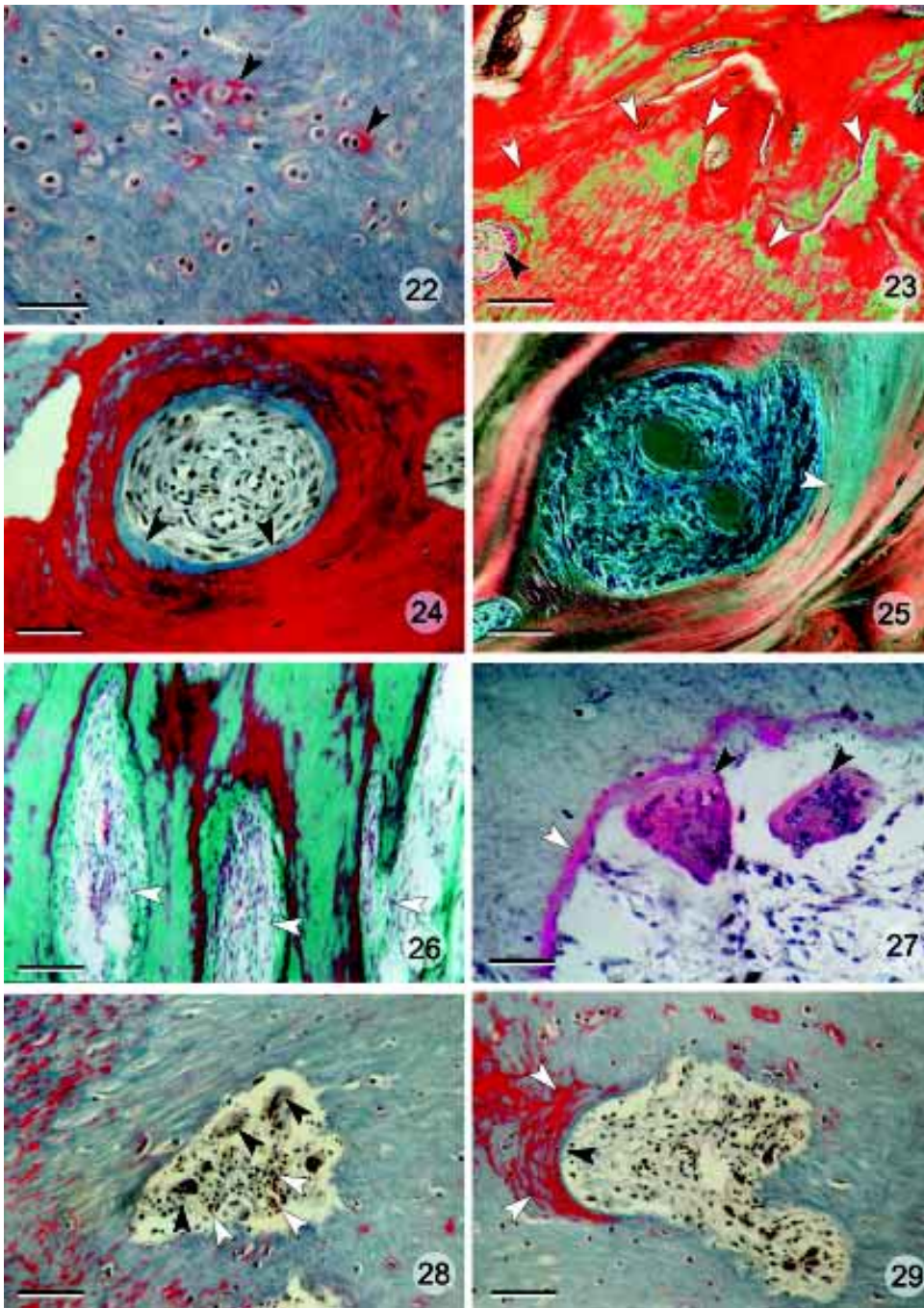


Fig. 22. Chondrocytes inside the skeleton of the kype mediate a cartilage-like mineralization (red staining) that starts in the vicinity of single cells and cell groups (black arrowhead). Pairs of chondrocytes indicate that cells continue to divide prior to matrix mineralization. Osteoid staining. Scale bar, 40 μ m.

Fig. 23. The compact bone of the dentary (top) is clearly separated from the skeleton of the kype (below) which is composed of both Sharpey fiber and chondroid bone. The border between compact and Sharpey fiber bone is labeled by white arrowheads. An osteon (black arrowhead) is forming in the vicinity of the cortical bone. Masson's trichrome staining. Scale bar, 150 μ m.

Fig. 24. A primary osteon in the cortex of the dentary. Notice the absence of chondrocytes and Sharpey fibers in the bone matrix. The endosteal surface is covered with osteoid (blue staining, black arrowheads). Osteoid staining. Scale bar, 60 μ m.

Fig. 25. A primary osteon in the cortex of the dentary. The circular arrangement of the parallel fiber bone (white arrowheads) has been visualized using polarized light microscopy. HBQ staining. Scale bar, 60 μ m.

Fig. 26. Cross linkage of skeletal needles creates bone marrow space that primarily contains connective tissue (white arrowheads). Masson's trichrome staining. Scale bar, 150 μ m.

Fig. 27. Bone resorbing cells in salmon display typical features, also known from mammalian Osteoclasts. The cells express TRAP (red), are multinucleated and display a ruffled border (black arrowheads). Secreted TRAP appears as a red stripe at the bone surface (white arrowhead). The extracellular dephosphorylation of bone sialoprotein and osteopontin by TRAP is thought to be responsible for the observed detachment of the cells from the bone surface (Ek-Rylander et al., 1994) as is the case for the right osteoclast and for cells in Fig. 28. TRAP staining. Scale bar, 40 μ m.

Fig. 28. An alternative way of creating bone marrow space is by bone resorption by multinucleated osteoclasts (black arrowheads). Along with the osteoclasts, blood vessels (white arrowheads) are present in the bone marrow. Osteoid staining. Scale bar, 60 μ m.

Fig. 29. Remodeling of chondroid bone into compact bone, finally results in the formation of new osteons, made of circular arranged parallel fibered bone (white arrowheads). As in complete osteons (Fig. 24), the endosteal surface is covered by osteoid (black arrowhead). Osteoid staining. Scale bar, 60 μ m.

enous fiber bundles that extend from the connective tissue, in this case of the kype, into the new bone matrix. Large osteoblasts secrete osteoid into the gaps between the fibers (Figs. 17,18). As in the deposition of skeletal needles distally, osteoblasts develop into typical osteocytes and chondrocytes (Fig. 19). Consequently, the ventral segment of the kype is Sharpey fiber bone that also displays features of chondroid bone similar to that described by Beresford (1993) as chondroid bone type one. HBQ- and Elastin-staining provide evidence that the fibers that continue from the

connective tissue into the bone matrix are made of collagen. Elastin, recently reported to occur in fish bone (Miyake *et al.*, 2001) is restricted to the connective tissue part of the kype and to the walls of blood vessels (Fig. 20).

Mineralization

As revealed by osteoid- and Masson's trichrome-staining, the pattern of mineralization of the skeletal tissue of the kype varies according to type and location of the developing hard tissue.

Skeletal needles display a mineralization front that starts beneath the broad osteoid zone (Fig. 11). In the ventral Sharpey fiber bone, collagen fibers are the sites of first mineral deposition (Fig. 21). Cartilage and cartilaginous parts of the skeleton of the kype undergo a cartilage-like pattern of mineralization, starting in the vicinity of chondrocytes (Fig. 22).

Bone Matrix

The kype skeletal tissue is made from primary cancellous, chondroid-like bone distally (Figs. 11-15) and from Sharpey fiber bone with chondroid bone-like features ventrally (Figs. 17-22). These tissues clearly differs from the compact bone of the dentary, which consists of parallel-fibered bone, structurally organized in osteons (Figs. 23-25). Compact bone contains neither chondrocytes nor Sharpey fibers (Fig. 24). The border between the compact bone of the dentary and the skeletal tissue of the kype is characterized by an abrupt change of bone type. The chondroid-like bone and the chondroid-like Sharpey fiber bone in direct contact with the compact bone (Figs. 6,23). The abrupt structural changes allows cortical bone and skeletal needles of the kype to be discriminated in (Figs. 3,4).

Remodeling

The skeletal tissue of the kype displayed evidence of continuous growth at the time the salmon were caught. Growth includes

development of cross connecting distal skeletal needles into a spongiosa-like formation (Figs. 6,7) which creates small bone marrow spaces containing connective tissue (Fig. 26). Concurrently, proximal parts of the skeleton of the kype are converted into compact bone (Fig. 21), which involves the creation of bone marrow space by osteoclastic bone resorption (Figs. 27-29), but not by cross connection of bone needles. From the analysis of serial sections we interpret this as a typical bone remodeling sequence involving the resorption of primary bone by numerous multinucleated and TRAP active osteoclasts (Figs. 27,28) and it's replacement by secondary lamellar bone (Fig. 29). Lamellar bone then forms the inner wall of forming osteons. Through bone remodeling, both the skeleton of the kype and the bone of the dentary increase and grow.

Discussion

The morphological alterations observed in the lower jaws of Atlantic salmon migrating upstream from the Miramichi river match the morphological descriptions of the phenomenon provided by Day (1987), Tchernavin (1938a) and Kacem *et al.* (1999) for Atlantic salmon migrating in Britain, Scotland and France. Hence, there is reason to assume that the alterations in the jaws of males found in this study are typical for Atlantic salmon in other locations, irrespective of population or genetic background.

Despite precise descriptions of the reinitiation of bone growth and morphological changes by Tchernavin (1938a) and Kacem *et al.* (1999), the hard tissue(s) of the kype have not been analyzed in detail. Darwin (1909, p. 510) discusses it as a cartilaginous projection, citing Yarrell's *History of British Fishes* as the source. Day (1887) cites Gadow as having sectioned a kype, stained it with carmine, and described it as consisting "entirely of fibrous connective tissue, without any traces of cartilaginous cells in it." Day goes on: "Therefore the hook cannot be looked upon either as an outgrowth of the bones of the lower jaw, or as a sort of horny excrescence like horns, nails..." (p. 57). In the few subsequent studies that have commented on the tissue (none of which undertook any histological analysis), it has been regarded as bone (Tchernavin, 1938c, Persson *et al.*, 1998). Our analysis reveals that the hard tissues of the kype are neither cartilage nor typical bone and are certainly not keratin as seen in horns or nails but differs from the compact bone of the dentary. The composition of the unique skeleton of the kype, its growth patterns, and its possible functional significance are discussed below.

The Skeletal Tissues of the Kype

The special characters of the skeletal tissues of the kype and their divergence from the compact bone of the dentary can be seen on high resolution X-rays, a technique that we and some others now regularly apply to detect skeletal alterations in teleosts (Witten *et al.*, 1999b, Afonso *et al.*, 2000). The border between new bone of the kype and of the dentary is clearly discernible on X-rays. Histological sections allow visualization of the microanatomy of the structures and confirm the radiological observations that proximally, compact bone of the dentary switches abruptly into a needle-like skeletal structure. The future application of X-radiography to analyze changes in the lower jaws of salmon in the field will allow analysis of large numbers of animals, and, with multiple X-rays through time, allow skeletal changes in individual animals to be followed. The use of such

TABLE 2

THE CHARACTERISTICS AND DIFFERENCES BETWEEN THE SKELETON OF THE KYPE AND THE COMPACT BONE OF THE DENTARY

Histological features	Figures
Skeleton of the kype	
The matrix consists of primary cancellous bone	9,11
Rapidly growing skeletal needles anastomose, forming spongiosa-like bone	6,7
Ventrally, the skeleton is solid and contains Sharpey fibers	8,17,19,23
Apical, mineralization starts beneath the broad osteoid zone, rich in proteoglycans	9,10,11
Ventrally, Sharpey fibers are sites of the first mineral deposition	21
Apically and ventrally, typical osteoblasts secrete osteoid and become entrapped in the matrix	9,10, 18
Osteoblast inside the matrix develop into osteocytes or into chondrocytes	12,13,18,19
Osteocytes are characterized by their typical shape and cell processes	12
Chondrocytes are characterized by:	8,13,15,22
the presence of groups of cells	13,22
a rounded shape	13,19,22
the capability to divide	22
the lack of cell processes	13,22
secretion of extracellular cartilage matrix including chondroitin sulphate, hyaluronic acid and collagen type II	8,15, 16,19
a cartilage-like pattern of matrix mineralization	22
Proximal parts of the skeleton of the kype are subjected to remodeling into compact bone	28,29
Compact bone of the dentary	
Composed of lamellar bone	23,24,25
Structural units are typical osteons	24,25
Osteoid seams are small and the bone is heavily mineralized	24
Osteoblasts and osteoid are present at endosteal surfaces of osteons	24
Compact bone contains no chondrocytes or chondrocyte-like cells and no Sharpey fibers	23,24,25
The tip of the dentary grows by remodeling of the skeleton of the kype into compact bone	23, 27,28,29
Remodeling creates bone marrow spaces	27,28,29
Remodeling involves bone resorption by typical osteoclasts	27,28,29

Fig. 5 provides a schematic overview of the arrangement of the different tissues.

a non-invasive technique on live animals is highly desirable in view of the world-wide declining stocks of wild Atlantic salmon. At the same time, there is an increasing need to gather information about life history of the animals (Hutchings and Jones, 1998).

Apart from radiological and microstructural studies, histological and histochemical analyses confirm the special character of the skeletal needles of the kype: (1) The needles display a rapid periosteal skeletal growth that differs from periosteal bone formation; (2) A loose meshwork of bony trabeculae then forms; (3) Subsequently, the tissue exhibits a character that is intermediate between bone and cartilage with Sharpey's fibers ventrally and (4) Finally the skeleton of the kype is partly remodeled into compact bone without cartilage-like features and without Sharpey fibers.

Rapid skeletal growth in a life phase where the animals have stopped feeding and utilize as much as seventy percent of their energy reserves for metabolically expensive upstream migration, for gonad maturation, and for competition with other males on the spawning grounds (Fleming, 1996) is a remarkable event. It has been hypothesized that formation of the kype and the changes in bones of the skull that occur during migration requires resorption of minerals from the operculum, the vertebral column or the scales (Tchernavin, 1938b; Persson *et al.*, 1998; Kacem *et al.*, 2000). The growth pattern of the skeletal tissue of the kype suggests, however, that despite mineral support from other skeletal elements, the bony tissue is made from the least amount of material possible; the skeleton of the kype does not arise as a solid bone mass, but as a meshwork of anastomosing skeletal needles. The spongiosa-like structure of the resulting tissue likely provides a maximum of mechanical stability with a minimum of bone mass, as in spongiosa in the bone marrow of mammals (Parfitt, 1988).

The spongiosa-like periosteal bone tissue in the skull of other teleosts (hyperostotic bone) might serve a mechanical function or only play a role in mineral metabolism (Smith-Vaniz *et al.*, 1995; Meunier and Desse, 1986). The rapid and structured growth of the skeleton of the kype as revealed by prominent osteoblasts, a broad osteoid zone, deposition of proteoglycans and expression of cartilage characteristics (Huysseune and Verraes, 1990; Beresford 1993; Nah *et al.*, 2000), suggests that the skeleton of the kype provides a maximum of support in a physiological situation where somatic growth has stopped (Persson *et al.*, 1998). Although, bone resorption occurs proximally in the kype, there is no indication that the skeleton of the kype functions as a mineral reserve in anadromous animals, since this and other studies show that bone resorption is always linked to the concurrent formation of new bone (Witten *et al.*, 2000).

The Function of the Kype and Kype Skeleton

So far, only a few studies have focused on the possible function of the kype. Tchernavin (1938c) regards the kype as "no good to the fish, and seems to be somewhat absurd, preventing as it does the use of the strong curved teeth which appear at the same time" (p. 37). Witten and Hall (2001) suggested that the kype is not merely a by-product of hormonal and environmental alterations. The kype might function as a secondary sexual character, either for competing with other males, or to attract females (Fleming, 1996; Hutchings and Myers, 1987; Järvi, 1990). Darwin certainly thought so:

Male salmon have been observed fighting all day long [and that] special means of defense may be given through means of sexual selection, as the mane of the lion and the hooked jaw to the male

salmon; for the shield may be as important for victory, as the sword or spear (Darwin, 1910, p. 64).

Ideas raised about functional and mechanical properties of the skeletal tissues of the kype are based on analysis of the hard tissue microstructure (Smith-Vaniz *et al.*, 1995). Thus, the unique character of kype hard tissues should be discussed in more depth. The skeleton of the kype arises from the periosteum of the dentary but its structure is clearly divergent from compact bone of the lower jaw, which is made of parallel-fibered bone and osteons. In contrast, the skeleton of the kype consists of anastomosing skeletal needles made of woven bone and contains no osteons. The differences between the kype and the compact bone of the dentary are summarized in Table 2. Besides osteocytes, the bone matrix contains chondrocytes, while the ventral part of the skeleton of the kype also contains Sharpey fibers.

Antlers of deer are periosteal bone formations that share features with the skeleton of the kype. Antlers function as a secondary sexual character, arise from a specific site on a dermal skeletal element (the pedicel of the frontal bone), and both their structure and mode of formation differ from those of the frontal bone. There is an initial cartilaginous phase and metaplastic conversion of cartilage to bone in antlers. Rapid growth and the retention of cartilage characters are features shared with the skeleton of the kype (Hall, 1978; Goss, 1983; Szuwart *et al.*, 1995, 1998).

In view of the astounding variety of skeletal tissue that occur in teleosts (Hall, 1978; Benjamin, 1988a,b; 1990; Huysseune, 2000) it is no surprise that secondary periosteal skeletal tissue is not only found in mammals and salmon but occurs in other fish species. Regular hyperostotic teleost bones — formerly regarded as pathological and referred to as an osteoma (Schlumberger and Lucke, 1948) — share features with the skeleton of the kype. Hyperostotic bones not only have a spongiosa-like appearance, but in teleosts with acellular bone they may represent areas of the skeleton that undergo remodeling (Smith-Vaniz *et al.*, 1995). In contrast, salmon have cellular (osteocyte-containing) bone and the whole skeleton (endoskeleton plus dermal bones and scales) is thought to be metabolically active (Persson *et al.*, 1998, 1999; Vielma and Lall, 1998; Kacem *et al.*, 2000). However, like hyperostotic bones of advanced teleosts (Smith-Vaniz *et al.*, 1995), in the upstream migrating salmon we examined, the skeletal tissue of the kype, not the compact bone of the dentary, displays remodeling, mediated by prominent multinucleated osteoclasts.

Bone and Cartilage Characters of the Kype Skeleton

The skeleton of the kype is formed by cells that resemble typical osteoblasts (Hall, 1978, 1990, 1991). In the ventral part, the cells secrete new bone matrix (osteoid) in gaps between the Sharpey fibers. Distally, membrane bone formation takes place. The two halves of the kype are united by hyaline cartilage, whose location and lack of connection to Meckel's cartilage would lead us to identify the tissue as secondary cartilage (Beresford, 1981). However, we do not yet know the developmental history of this cartilage. It could be a remnant of Meckel's cartilage not connected to Meckel's cartilage in adults; it would have to arise from periosteal cells to qualify as a secondary cartilage (see Benjamin, 1988b).

Interestingly, not all cells that derive from osteoblasts and become entrapped in the bone matrix develop into osteocytes. Cells that have all the features of osteoblasts also develop into typical chondrocytes, the latter identified as being rounded, show-

ing cell division, being arranged in rows and territories, producing cartilage matrix and displaying a cartilage-like pattern of matrix mineralization (Moss and Moss-Salentijn, 1983; Huyseune, 2000). Given that the skeleton of the kype entirely derives from intramembranous ossification, the appearance of cartilage characters is remarkable. However, it is now established that the expression of cartilaginous features is a general character of growing membrane bones (Fang and Hall, 1997, 1999; Nah *et al.*, 2000). Furthermore, there is strong evidence that osteoblasts and chondroblasts are members of the same class of scleroblasts (Hall, 1970, 1978, 1991; Hall and Miyake, 2000). The mechanisms discussed above are likely also involved in the development of chondroid bone, a tissue that occurs in several teleost species and that shares morphological and histochemical features with the skeleton of the kype (Beresford, 1981; Benjamin, 1990; Huyseune and Verraes, 1990; Taylor *et al.*, 1994). Similar to chondroid bone in cichlids, the growth of the kype bone involves osteoblasts that acquire characteristics of chondrocytes as they become trapped in the bone matrix. The idea has been raised that chondroid bone is an adaptation to rapid bone growth (Hall, 1990; Huyseune, 1990; Taylor *et al.*, 1994), an interpretation that applies to both the distal and the ventral parts of the skeleton of the kype.

Sharpey Fiber Bone in the Kype Skeleton

Apart from displaying chondroid bone-like characters, the ventral part of the skeleton of the kype is also a Sharpey fiber bone. A similar tissue, that displays features of chondroid bone and of Sharpey fiber bone, has not been described in teleosts previously. Sharpey fiber bone - characterized by the continuation of collagen fiber bundles from connective tissue into the bone matrix - is commonly associated with joints and teeth, providing a strong link between connective tissue and the skeleton (Beresford, 1981; McKee *et al.*, 1996). Hence, the presence of Sharpey fiber bone in the ventral part of the skeleton of the kype further supports the idea that this skeletal tissue is functionally integrated in the architecture of the salmon jaw. It is not a by-product of physiological changes but serves a mechanical function.

Appearance of Sharpey fiber bone in the dentary is not restricted to the kype of grilse. Hughes *et al.* (1994a,b) described Sharpey fiber bone in the lower jaws of juvenile salmon. Interestingly, their description of Sharpey fiber bone in the lower jaw of perciform teleosts (Sparidae) also matches the tissue found in salmon. In distinction from Hughes *et al.* (1994b), who identified all cells inside the Sharpey fiber bone as osteocytes, we conclude that the Sharpey fiber bone of salmon contains chondrocytes. If the cells inside the Sharpey fiber bone of Sparidae that display a cartilage-like morphology are chondrocytes and not osteocytes (as we suspect from the figures published by Hughes *et al.*, 1994b), then the type of hard tissue in the kype ventrally is not restricted to salmon.

The Kype and Salmon Life History

The consequences of different life strategies of male Atlantic salmon that range from premature kype-less pair to kype-bearing mature male salmon are not fully understood (Shear, 1992; Moore and Waring, 1999; Jones and Hutchings, 2001). Mature males invest heavily in the spawning success, and the development of a prominent kype is a part of the male investment, resulting in an increased mortality (Fleming, 1996). As suggested by Tchernavin

(1938c), the kype may become a disadvantage for anadromous males (impairing proper feeding, breathing and swimming) after fulfilling its purpose as a secondary sexual character. Although, the microstructure of the kype tissue is different from the bone of the jaws, developmental processes and hard tissue elements of the kype are known from the dermal skeleton of mammals and other teleost species. Finally, the rapid growth of the skeleton of the kype appears to be a regulated process, integrated in the development of the dentary, and understandable in the light of the general strategy of Atlantic salmon to survive spawning. The growth of compact bone, through remodeling of the skeleton of the kype seems to produce non-reversible alterations of the dentary in repetitively spawning individuals. However, it remains to be clarified if the changes gradually disappear after spawning and if the dentary resumes its former shape and proportions in those males which return to sea, as hypothesized by Tchernavin (1937). Future studies will address the extent of the process and its limits for the fitness of individual anadromous male salmon.

Materials and Methods

Sampling

Ten male anadromous grilse (see Table 1 for definition of terms), collected during the fall run in 1999 and 2000 (September) at the south-west Miramichi River above Red Bank (46°56.8' N, 65°48.3' W, New Brunswick, Canada,) were used for this study. Animals were caught in traps used for the Red Bank (First Nation) food fishery. The animals were caught and sampled under license from the Canadian Federal Department of Fisheries and Oceans.

Weight and fork length were determined in order to calculate the condition factor ($K = [\text{weight (g)/fork length (cm}^3)] \times 100$) of the fish (Arndt *et al.*, 1996). Scales were taken to evaluate the animals life history; part of a Department of Fisheries and Oceans monitoring program. All grilse used for this study were in "good shape," as determined by gross morphological inspection, and showed no signs of diseases. As revealed by scale reading (Shearer, 1992), all animals had spend one winter in the ocean (one sea winter salmon = 1SW; see Table 1) before returning to their home river. The fork length of males varied between 55.4 and 63.5 cm, weight ranged from 2010g to 2500g. Condition factors were calculated as between 0.93 and 1.17 which is in the range of the mean condition factor ($K = 1.02$) for males from this age group in the Miramichi river system (Moore *et al.*, 1995).

For gross anatomical documentation external picture of the entire head as well as pictures of half heads were taken. Subsequently, the jaws were x-rayed, processed and immediately fixed for seven different analytical procedures.

X-ray Analysis

High resolution X-rays of the left halves of all fish heads were taken using a portable "Mini X-ray HF80+" machine (Mini X-ray inc., Northbrook, USA) and "Kodak Industrex M Film Ready Pack II" (Kodak Industry, France) without a screen. The settings of the X-ray unit were 70 kV, 15 mA, 2 sec exposure time, and a distance of 40 cm between the beam source and the X-ray film. Radiographs were developed with Kodak chemicals and analyzed using a stereo microscope.

Basic Histological Procedures

Immediately after capture, right jaws were fixed in 10% neutral buffered formaldehyde for 24 hrs, rinsed in tap water for 24 hrs, and decalcified for 72 hours in a 10% EDTA solution buffered with 0.1 M TRIS base, pH 7.0. After decalcification, samples were dehydrated in a graded series of ethanol and subsequently embedded in Paraplast.

Starting at the midline 10 μm sagittal sections were prepared and stained with Masson's Trichrome (Flint and Lyons, 1975) for demonstration of collagen, bone cells, and mineralized bone and with the Hall-Brunt

Quadruple Stain (HBQ) (Hall, 1986) to differentiate bone, cartilage, chondroid bone and to identify collagen fibers. Sections were mounted with DPX (Fluka).

Additional Histological and Histochemical Analyses

Additional analytical procedures were applied to demonstrate osteoid and the pattern of mineralization, visualize bone remodeling, identify chondrocytes and cartilage matrix within bone, and to discriminate among elastin and collagen fibers.

To differentiate osteoid (non-mineralized bone matrix) and mineralized bone matrix and to reveal the pattern of mineralization, osteoid staining was performed, following the trichrome method of Ralis and Watkins (1992).

To demonstrate bone remodeling, decalcified, sections were stained with Toluidine Blue as follows: Descending ethanol series (100% ethanol to water); 1% aqueous Toluidine Blue solution (pH 4.5) for 45 minutes; differentiation and dehydration in 100% Ethanol; mounting.

To identify osteoclasts and osteoclasts activity, tartrate resistant acid phosphatase was demonstrated (TRAP) following the protocol of Witten *et al.*, 2001. Briefly, samples were (a) fixed in 10% formaldehyde (buffered with 50 mmol l⁻¹ TRIS, at pH 7.2; (b) rinsed in tap water for 1 hr; (c) decalcified with EDTA (see above), (d) dehydrated in graded acetone solutions and (e) embedded in glycol-methacrylate. Sections of 5 µm were mounted on uncoated slides. For demonstration of TRAP activity, specimens were preincubated for 30 min at 20°C in 100 mmol acetate buffer + 50 mmol L(+) di-sodium tartrate dihydrate at pH 4.5. Staining was performed in the presence of tartrate using naphthol AS-TR phosphate as substrate (final concentration 170 mmol/L) and hexazotised pararosaniline (final concentration 1.58 mmol/L) as an azo-coupling dye. Specimens were counterstained with Mayer's haematoxylin. Control procedures revealed the specificity of the staining and consisted of: (a) heating at 90°C for 10 min prior to staining, (b) incubation without substrate, (c) adding the inhibitor NaF to the staining solution (10 mmol l⁻¹).

For the selective demonstration of cartilage matrix proteoglycans, sections were stained with a combined Alcian Blue/Haematoxylin staining (AH). Deparaffinized sections were treated with 3% acetic acid for 3 min, 1% Alcian Blue in 3% acetic acid solution for 1 hr, rinsed in 3% acetic acid solution, rinsed for 10 min in D H₂O, stained with Myer's acid Haematoxylin for 10 min, flushed in running tap water for 10 min and then mounting.

Cartilage-specific proteoglycans were visualized by selective removal of two cartilage matrix components prior to Alcian Blue Haematoxylin staining: hyaluronic acid, which was digested with hyaluronidase, and chondroitin-6-sulphate, which was digested with chondroitinase. To remove hyaluronic acid, sections were incubated for 30 min at 37°C in a solution containing 6 mg/ml hyaluronidase (EC. 3.2.1.35, Sigma No. He506) dissolved in 10 mM PBS buffer (pH 7.2). To remove chondroitin-6-sulphate, prior to staining, sections were incubated at 37°C in a solution containing 5 units/ml of Chondroitinase ACB (chondroitin-6-sulphatase; E.C. 4.2.2.4., Sigma No. C2905) in 10 mM TRIS buffer (pH 8.0).

Immunostaining for collagen type II as a component of the cartilage matrix was performed on decalcified specimens embedded in Paraplast (see histology). After removal of paraffin, sections were pre-treated with hyaluronidase following the protocol given above to expose collagen. As part of the protocol, the solution also contained 10 mM Levamisole to inactivate alkaline phosphatase (levamisole treatment may be omitted since decalcification with EDTA also inactivates alkaline phosphatases). Sections were then incubated in a blocking solution for 30 minutes at 37°C (20% Rabbit Serum in 10 mM PBS + 10 µM Levamisole, pH 7.4). Subsequently a monoclonal anti-collagen type II antibody (mouse IgG; II-I16B3, Developmental Studies Hybridoma Bank, Iowa USA) was used to identify cartilage matrix collagen. 100 ml antibody containing supernatant and 10 ml rabbit serum were dissolved in 890 ml PBS and applied to the sections at 4°C for 12 hrs. Slides were flushed with PBS buffer and the sections were treated with the secondary antibody for 30 min at 37°C (a ALP-labelled anti-mouse IgG raised in rabbits, anti-mouse IgG/PBS 1:50 + 10% Rabbit serum). Finally the sections were flushed two times with PBS, pre-adjusted pH 9 with TRIS-buffer and ALP was visualised by BICP/NBT

staining for 30 min at 20°C. Control procedures revealed the specificity of the staining and consisted of: (a) incubation with a non-specific mouse IgG antibody, (b) incubation only with the alkaline phosphatase-labelled secondary antibody, and (c) incubation with ALP substrate only.

Since elastin was recently described as a component of bone and teeth in fish (Miyake *et al.*, 1999, 2001) the possible presence of elastin fibers in the hard tissues of the kype was evaluated using the Verhoeff staining procedure (Presnell and Schreiber, 1997).

Acknowledgements

For discussions on the topic of this study we thank Harald Rosenthal, Tom Miyake, Lawrence Taylor, Gérald Chaput, Michel Chadwick, David Moore, Peter Hurlley, John Hayward and Joe Sheasgreen. We are grateful to Rick Cunjak who facilitated access to field facilities at the start of this study. We thank people from the Red Bank (First Nation, New Brunswick, Canada), especially Betty Jane Ward for sampling salmon. We also appreciate support provided by Wolfgang Villwock, Volker Hilge, Thomas Falk, and Gudrun Schulze. Funding for this study was granted by the German-Canadian Science and Technology Cooperation, by the Deutsche Forschungsgemeinschaft (DFG) and by the Natural Sciences and Engineering Research Council of Canada (NSERC).

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Received: February 2002

Reviewed by Referees: April 2002

Modified by Authors and Accepted for Publication: July 2002