Transactions of the American Fisheries Society 110:708–717, 1981

Method to Evaluate Mechanical Properties of Bone in Fish

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Abstract

A method for testing the mechanical properties of fish vertebrae was adapted from other vertebrae studies and used to describe the structural integrity of fishes' vertebra tissues. Individual vertebrae from the anterior region of the spinal column were subjected to compression tests to determine strength (ultimate stress and stress at elastic limit), elasticity (ultimate strain and modulus of elasticity), and energy-absorbing capacity (toughness). For brook trout *Salvelinus fontinalis,* channel catfish *Ictalurus punctatus,* and bluegills *Lepomis macrochirus,* mechanical properties of bone differed among species and changed with age within a species. Overall, changes in mechanical properties were related to age-dependent changes in bone density.

Structural integrity of the backbone is a critical element in a fish's ability to survive because the spinal column is the key support structure for muscle attachments used in swimming (Gray 1957). Structural integrity is described by mechanical properties such as strength, elasticity, and energy-absorbing characteristics (Evans 1973). Mechanical testing techniques have been used on animals to evaluate mechanical properties of bone (Weir et al. 1949; Rucker et al. 1975), skin (Nimni and Bavetta 1965; Seifter et al. 1975), and tendon (Rigby 1964; Oakes and Bialkower 1977). However, the mechanical properties of fish tissue have come under investigation only recently (Yoshinaka et al. 1977; Lauder and Lanyon 1979).

In this report we describe development of a method to evaluate mechanical properties of fish vertebrae as an indicator of backbone structural integrity. Mechanical properties of bone usually are tested on whole bone or standardized test specimens of bone tissue (Currey 1970; Evans 1973). Mechanical properties of bone in all animals depend on the quality (composition) and quantity (density) of bone material. As a young animal grows to adulthood, changes in composition and density of bone occur that alter its structural integrity and mechanical properties (Bartley et al. 1966; Bell et al. 1967; Vogel 1979). To adapt mechanical testing techniques from avian and mammalian studies to fish tissue, we selected whole vertebrae from fish for testing. The mechanical properties of vertebrae were determined for various life stages of three species: brook trout *Salvelinus fontinalis,* channel catfish *Ictalurus punctatus,* and bluegill *Lepomis macrochirus.* Biochemical characteristics of composition and density were determined for the vertebrae to evaluate their relations to mechanical properties of bone.

Methods

Test Species

Fish were maintained at the Columbia National Fisheries Research Laboratory, Columbia, Missouri, in well water (temperature, 15–17 C; pH, 7.2–7.4; alkalinity, 237 mg/liter as $CaCO_3$; hardness, 272 mg/liter as $CaCO_3$) and fed Rangen's commercial diet ad libitum (Brauhn and Schoettger 1975).

Three-month-old brook trout fry, obtained from Big Spring Trout Hatchery, Lewistown, Montana, were randomly sampled and tested at ages of 9, 12, 13, 15, and 19 months.

Channel catfish raised from eyed eggs supplied by Tishomingo (Oklahoma) National Fish Hatchery were randomly sampled and tested at ages of 6, 12, and 15 months. Three-month-old channel catfish were obtained for testing from Ozark Fisheries, Osage Beach, Missouri. Two-year-old adults, obtained from Schroeder's Fish Farm, Carlisle, Arkansas, were separated by sex to determine differences between sexes in bone properties.

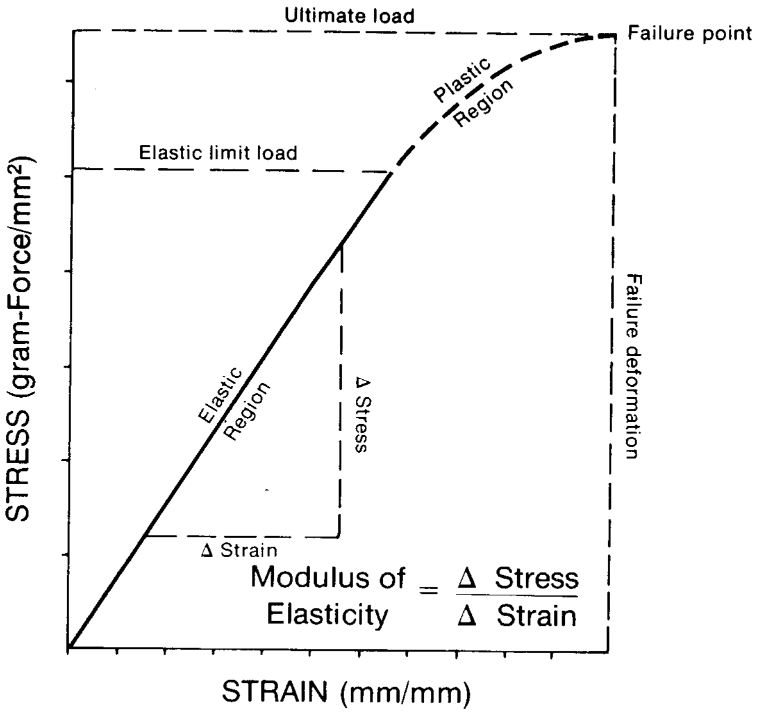

FIGURE 1.—*Generalized stress-strain curve for all types of materials, including vertebrae, under constant rate of compression strain.*

TABLE 1.—*Mechanical properties, biochemical composition, and density of vertebral bone of brook trout of various sizes and ages. The values represent the mean of fish within an age. Values in the same row with letters in common are not significantly different (P > 0.05).*

Characteristics	Age of fish (months)				
	9	12	13	15	19
Mechanical properties					
Rupture (g-force/mm^2)	779 a	890 ab	1,054 b	927 ab	1,002 b
Elastic limit (g-force/mm^2)	642 a	730 ab	932 c	753 ab	814 bc
% strain	18.7 a	30.6 b	25.3 c	21.5 a	21.9 a
Modulus of elasticity (kg-force/mm^2)	6.90 a	5.00 b	5.31 bc	6.17 ac	5.43 bc
Toughness (g-mm/mm^3)	96 a	184 b	155 bc	125 ac	122 ac
Biochemical composition					
Collagen (mg/g)	251 a	240 b		233 b	255 a
Hydroxyproline (mg/g collagen)	68.2 b	62.7 b		85.8 c	75.6 d
Phosphorus (mg/g)	114.4 a	91.6 b		104.0 bc	97.9 bc
Calcium (mg/g)	200.7 ab	172.2 c		185.1 bc	209.0 a
(Ca + P)/collagen	1.3 a	1.1 b		1.2 ab	1.2 ab
Density (mg/cm^3)	508 a	557 b		555 b	600 c
Fish size and number					
Length (mm)	129 a		233 b	267 c	294 d
Weight (g)	25.9 a		168.2 b	269.7 c	323.9 c
Number of fish	8	10	6	6	8

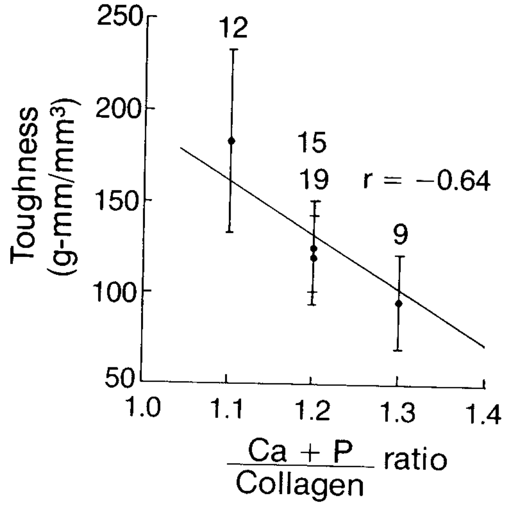

FIGURE 2.—*Relationship between vertebral energy-absorbing capacity (toughness) and bone mineral : collagen ratio in brook trout 9, 12, 15, and 19 months old. Values are means ± 1 SD for each age.*

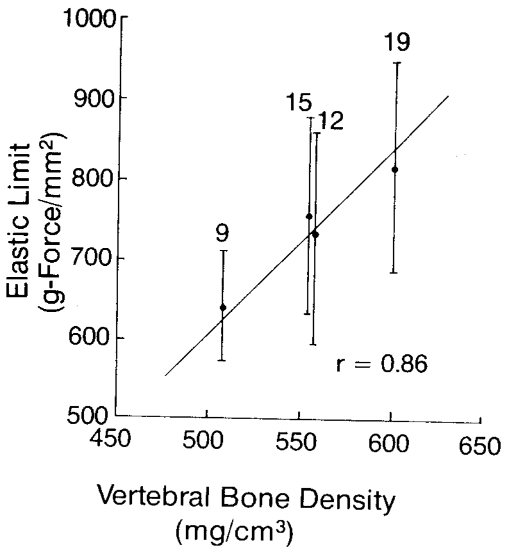

FIGURE 3.—*Relationship between vertebral strength (elastic limit) and bone density in brook trout 9, 12, 15, and 19 months old. Values are means ± 1 SD for each age.*

One-month-old bluegill fry, obtained from Corning (Arkansas) National Fish Hatchery, were sampled and tested at 2 and 9 months of age; 12-month-old fish tested were obtained from Ozark Fisheries.

Preparation and Mechanical Testing of Vertebrae

Measurements of weight (g) and total length (mm) of each fish were recorded before dissection. The second through ninth vertebrae posterior to the skull were dissected from brook trout and bluegills. From channel catfish, the seventh through 15th vertebrae posterior to the complex-vertebrae structure were taken. Spines and soft tissue were removed from the vertebral body, leaving only the centrum. Individual vertebrae were measured with an ocular micrometer and microscope for 9-month-old brook trout, 3- and 6-month-old channel catfish, and 2- and 9-month-old bluegill, and with a micrometer (measurements to nearest 0.05 mm) for all other ages to determine anterior-posterior length (l), and mean radius (R = [lateral diameter + dorsal-ventral diameter] ÷ 4). These measurements were used to calculate the cross-sectional area ($A = \pi R^2$) and volume ($V = A \times l$) on which compressional forces act during loading. After dissection and measurement, vertebrae were dampened with distilled water, placed in capped test tubes, and stored at 4 C up to 24 hours before they were tested. Vertebrae were brought to room temperature over a 15–30-minute period before testing began.

The effects of three tissue-storage techniques—preservation in 10% buffered neutral formalin or in ethylene glycol, and storage of whole fish at low temperature (−20 C)—on the mechanical properties of fish vertebrae were examined to determine the best way for preserving bone tissue for later mechanical testing.

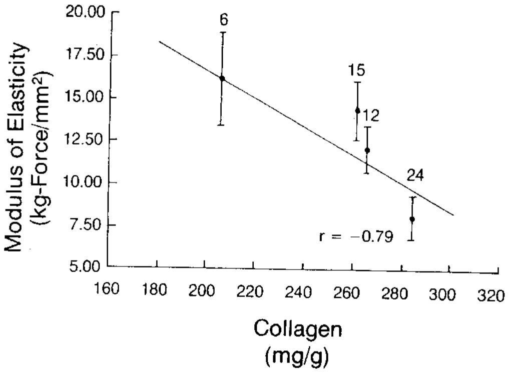

FIGURE 4.—*Relationship between vertebral elasticity (modulus of elasticity) and bone collagen concentration in channel catfish 6, 12, 15, and 24 months old. Values are means ± 1 SD for each age.*

TABLE 2.—*Mechanical properties, biochemical composition, and density of vertebral bone of channel catfish of various sizes and ages. The values represent the mean of fish within an age. Values in the same row with letters in common are not significantly different (*P *> 0.05).*

Characteristics	Age of fish (months)				
	3	6	12	15	24+
Mechanical properties					
Rupture (g-force/mm²)	1,079 a	1,486 b	1,812 c	1,341 bd	1,180 ad
Elastic limit (g-force/mm²)	753 a	1,107 b	1,247 b	916 a	899 a
% strain	17.9 a	14.2 b	21.2 c	18.5 ac	25.2 d
Modulus of elasticity (kg-force/mm²)	8.92 a	16.17 b	12.07 c	14.37 bc	8.04 a
Toughness (g-mm/mm³)	114 a	133 ab	231 c	166 bd	195 cd
Biochemical composition					
Collagen (mg/g)	335 a	204 b	263 c	259 c	283 c
Hydroxyproline (mg/g collagen)	31.5 a	73.6 b	76.8 b	72.5 b	90.8 c
Phosphorus (mg/g)	109.0 a	96.1 b	86.1 b	101.7 a	102.7 a
Calcium (mg/g)	229.1 a	179.8 b	163.5 b	221.6 ac	202.3 c
(Ca + P)/collagen	1.0 ac	1.4 b	0.9 a	1.3 b	1.1 c
Density (mg/cm³)	772 a	823 a	819 a	639 b	601 b
Fish size and number					
Length (mm)	94 a	98 a	142 b	234 c	495 d
Weight (g)	8.9 a	8.5 a	54.3 ab	246.4 b	1,142.6 c
Number of fish	8	9	6	7	8

Preservation with either buffered formalin or ethylene glycol altered mechanical properties of bone by artificially stiffening and strengthening vertebral tissue, but low-temperature storage had no effect. These respective effects are similar to those observed by Evans (1973) for preservation of human bone. Consequently, mechanical properties were determined only for fresh or frozen bone.

Mechanical properties of vertebrae were determined by the compression loading of individual vertebrae aligned in an anterior-posterior direction with the compressive force. Preliminary mechanical testing revealed that vertebrae of the posterior trunk and anterior caudal areas were the strongest and stiffest, anterior trunk vertebrae had intermediate strength and stiffness, and posterior caudal vertebrae had low strength and stiffness. Consequently, testing was performed on anterior trunk vertebrae. Preliminary mechanical testing of vertebrae also revealed that fresh (wet) vertebrae gave more consistent results than did vertebrae dried to a constant weight at 100 C. Dry bone generally is not used in mechanical tests because it behaves like inorganic material, whereas wet bone behaves similar to bone in vivo (Evans 1973), so we used wet vertebrae. Preliminary mechanical testing results revealed that eight vertebrae per fish and six to eight fish per group would be needed to keep variation of the standard error around the mean of mechanical-property data within 15% for fish of each age.

Compression loading was performed with Instron testing machines Model 1122 (English units) or Model 1132 (metric units). The crosshead of the machines moved at a constant closure rate of 5.1 mm per minute for Model 1122 and 5 mm per minute for Model 1132. Bone tissue is load-rate sensitive (McElhaney 1966), and the 1.6% difference in crosshead speeds of the two Instron machines resulted in a significant difference (analysis of variance, $P = 0.05$) in the mechanical properties. Mechanical testing results from the two machines were considered separately.

We used a typical stress-strain curve resulting from vertebral resistance to a constant-strain compressive load to calculate the mechanical properties (Fig. 1). Mechanical properties describing strength include ultimate stress, which identifies the force level causing specimen failure, and stress at the elastic limit, which identifies the force level above which permanent structural damage occurs in a test specimen. The elastic region of the stress-strain curve is where a test specimen shows a linear relationship between strain and stress. The plastic re-

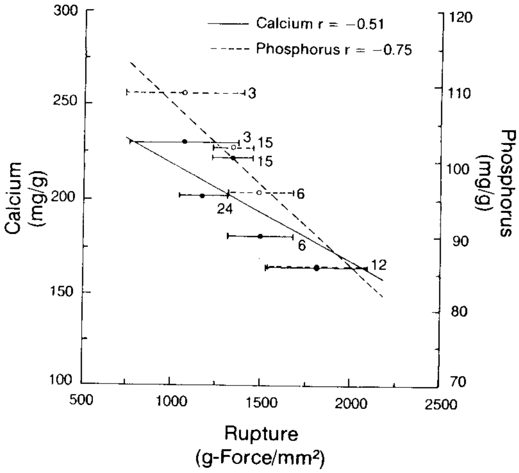

FIGURE 5.—*Relationship between vertebral strength (rupture) and bone mineral components, calcium and phosphorus, in channel catfish 3, 6, 12, 15, and 24 months old. Values are means ± 1 SD for each age.*

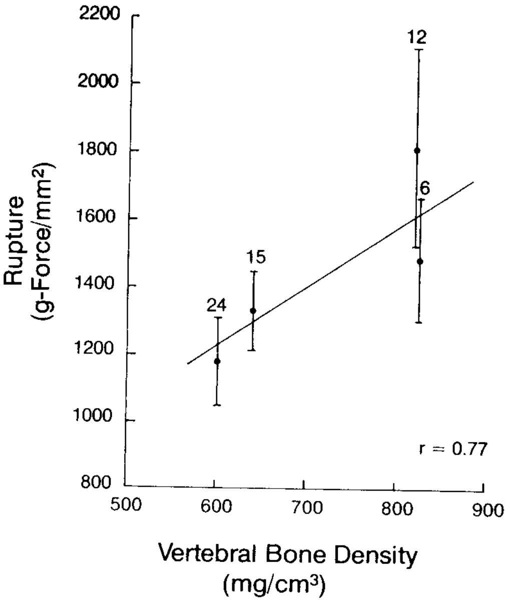

FIGURE 6.—*Relationship between vertebral strength (rupture) and bone density in channel catfish aged 6, 12, 15, and 24 months. Values are means ± 1 SD for each age.*

gion of the stress-strain curve is where a test specimen no longer shows a constant stress-strain proportionality.

$$\text{Ultimate stress} = \frac{\text{ultimate load}}{\text{cross-sectional area of vertebra in contact with load}}.$$

$$\text{Stress at elastic limit} = \frac{\text{load at elastic limit}}{\text{cross-sectional area of vertebra in contact with load}}.$$

Mechanical properties describing elasticity include ultimate strain (percent), which is the amount of deformation a test specimen incurs at the failure point, and modulus of elasticity (ME), which indexes the stiffness of a test specimen.

$$\text{Ultimate strain (\%)} = \frac{100 \times \text{(change in vertebral length at failure)}}{\text{original anterior-posterior length}}.$$

Modulus of elasticity = slope of elastic region of stress-strain curve (stress per unit strain in elastic region).

Strength and elasticity characteristics interact to determine toughness, which is the amount of force and deformation absorbed by a test specimen before it fails.

Toughness = area beneath stress-strain curve preceding failure point (total work required to break specimen).

Vertebral failure was judged to occur when the test specimen showed a "breaking" peak or pure plastic deformation, that is, a change in strain without a change in stress, on the stress-strain curve. Hereafter, ultimate stress is referred to as rupture, stress at elastic limit as elastic limit, and ultimate strain as percent strain.

Biochemical Composition Analyses and Density Formulations of Bone

After they were mechanically tested, vertebrae were dried to a constant weight at 100 C in a forced-air oven for 2 to 8 hours (depending on size—2 hours for 3-month-old channel catfish to 8 hours for adult channel catfish), split into two fractions, and weighed. Collagen (mg/g bone) was isolated from one fraction by the method of Flanagan and Nichols (1962). The isolated collagen was weighed and hydrolyzed

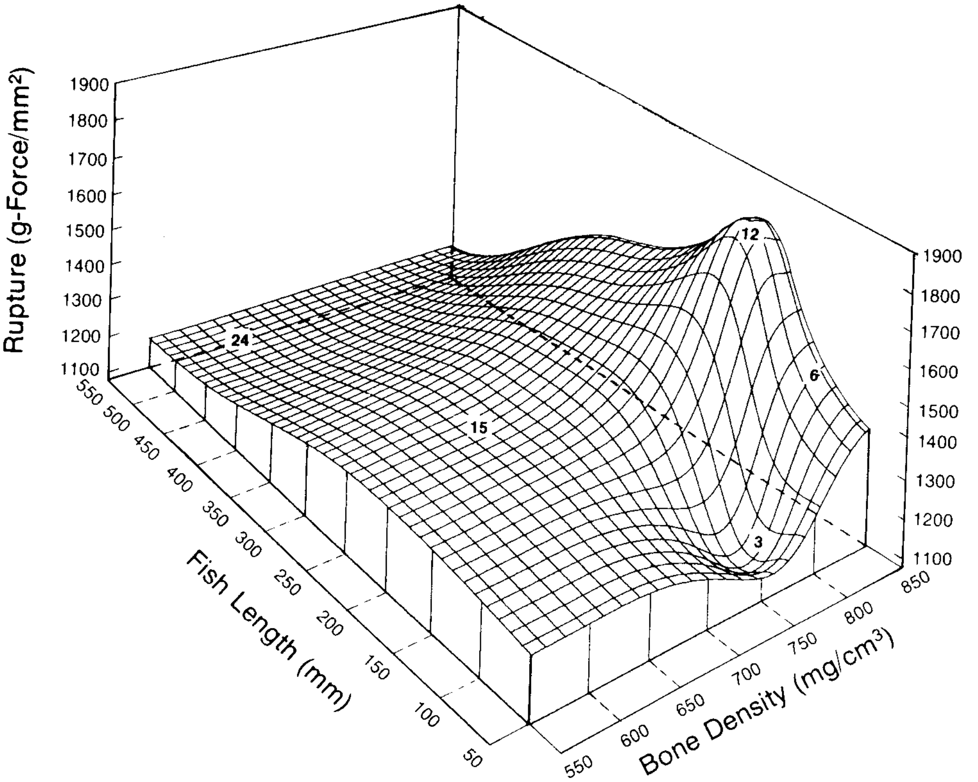

FIGURE 7.—*Relationship between vertebral strength (rupture), bone density, and fish length in channel catfish of various ages. Numbers on the response surface are fish ages (months).*

at 115 C in 2–5 ml of 6 N HCl (2 ml for brook trout, young channel catfish, and bluegills; 5 ml for adult channel catfish) for 16 hours. Hydroxyproline (mg/g collagen) was determined in a 2-ml sample (Woessner 1961). The other bone fraction was hydrolyzed at 115 C in 2–5 ml of 6 N HCl for 16 hours before calcium was determined by the method of Gitelman (1967) and phosphorus by the method of Fiske and Subbarow (1925).

Vertebral density was the mean dry weight of eight vertebrae weighed together divided by the mean volume of eight vertebrae measured individually.

Statistical Analyses

Vertebral properties (mechanical, biochemical, density) were averaged over all fish within a species and age, and subjected to analysis of variance. A multiple-means comparison test (least significant difference) assessed significant ($P \leq 0.05$) differences among groups (Snedecor and Cochran 1967).

Results

Brook Trout

The strength (rupture and elastic limit) and energy-absorbing capacity (toughness) of brook trout vertebrae followed a biphasic trend with age (Table 1). Elasticity of vertebrae remained relatively constant in the four oldest ages, but was reduced in 9-month-old brook trout; this was probably due to the stiffening effect of the high mineral content of the bone in relation to organic matrix (high mineral : collagen ratio).

Biochemical composition of bone remained nearly constant throughout the ages of brook trout examined. Organic matrix and mineral components of the backbone were not statistically correlated with the mechanical properties. The ratio mineral : organic matrix, however,

TABLE 3.—*Mechanical properties, biochemical composition, and density of vertebral bone of bluegills of various sizes and ages. The values represent the mean of fish within an age. Values in the same row with letters in common are not significantly different* (P > *0.05*).

Characteristics	Age of fish (months)		
	2	9	12
Mechanical properties			
Rupture (g-force/mm²)	999 a	808 ab	754 b
Elastic limit (g-force/mm²)	886 a	751 b	548 c
% strain	10.8 a	10.3 a	18.5 b
Modulus of elasticity (kg-force/mm²)	11.48 a	9.70 b	6.06 c
Toughness (g-mm/mm³)	63 a	49 a	85 b
Biochemical composition			
Collagen (mg/g)	245 a	240 a	256 a
Hydroxyproline (mg/g collagen)	34.3 a	37.1 a	106.7 b
Phosphorus (mg/g)	114.3 a	113.1 a	106.6 a
Calcium (mg/g)	166.3 a	203.4 b	208.6 b
(Ca + P)/collagen	1.1 a	1.4 b	1.3 ab
Density (mg/cm³)	635 a	513 b	720 c
Fish size and number			
Length (mm)	41 a	56 b	100 c
Weight (g)	1.5 a	3.7 a	19.9 b
Number of fish	8	8	8

was negatively correlated with energy-absorbing capacity of the bone (toughness $r = -0.64$, Fig. 2). This result supports the hypothesis of Mayer et al. (1977) that an increase in the mineral : organic-matrix ratio in fish vertebrae could indicate a weak, brittle backbone.

Bone density increased between 9 and 12 months of age and between 15 and 19 months of age. Increased bone density in brook trout vertebrae was positively correlated with increases in vertebral strength (rupture $r = 0.76$ and elastic limit $r = 0.86$, Fig. 3).

Channel Catfish

The strength (rupture and elastic limit) and energy-absorbing capacity (toughness) of channel catfish vertebrae also followed a biphasic response with age (Table 2). Vertebrae from 3- and 24-month-old fish were less strong and more elastic (decreased ME) than those of the other ages. Twelve-month-old fish had the greatest elastic limit and rupture values, and therefore the strongest vertebrae. These mechanical properties are similar to those in human vertebrae, which increase in strength during adolescence, are greatest between 20 and 30 years of age, and then decline with increasing age (Bartley et al. 1966; Rockoff et al. 1969).

Increased collagen concentrations in fish backbone increased the elasticity of vertebrae (ME $r = -0.79$, Fig. 4); the negative correlation indicates a positive physical relationship between collagen concentration and vertebral elasticity. Rucker et al. (1975) described similar relationships in femurs of chicks fed copper-deficient diets.

Mineral components of bone were related to force-related mechanical properties; rupture increased as concentrations of calcium ($r = -0.51$) and phosphorus ($r = -0.75$) decreased (Fig. 5) and, similarly, elastic limit increased as calcium ($r = -0.54$) and phosphorus ($r = -0.62$) concentrations decreased. These results are similar to those of Currey (1969), who showed that mineralization of bone, as measured by ash content, strongly influenced the mechanical strength. He demonstrated that bone strength increases with increasing mineral deposition at low mineralization levels, and is reduced with increasing deposition at high levels. Furthermore, Currey concluded that the advantage of having more mineral in bone to bear a load becomes counterbalanced by the tendency of cracks to run further through the solid mineral phase, thus causing a bone to fail at lower force levels.

Vertebral bone density also followed a bi-

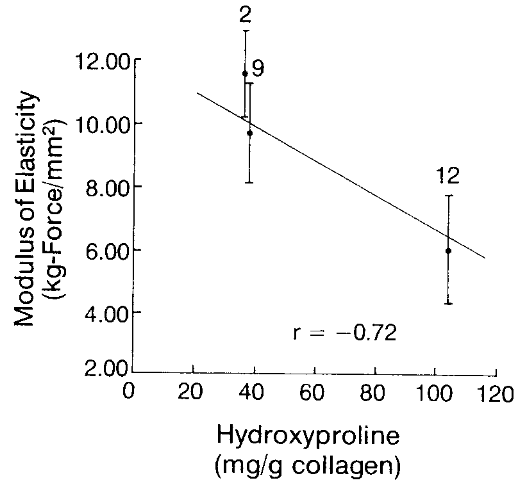

FIGURE 8.—*Relationship between vertebral elasticity (modulus of elasticity) and hydroxyproline concentration in backbone collagen of bluegills aged 2, 9, and 12 months. Values are means ± 1 SD for each age.*

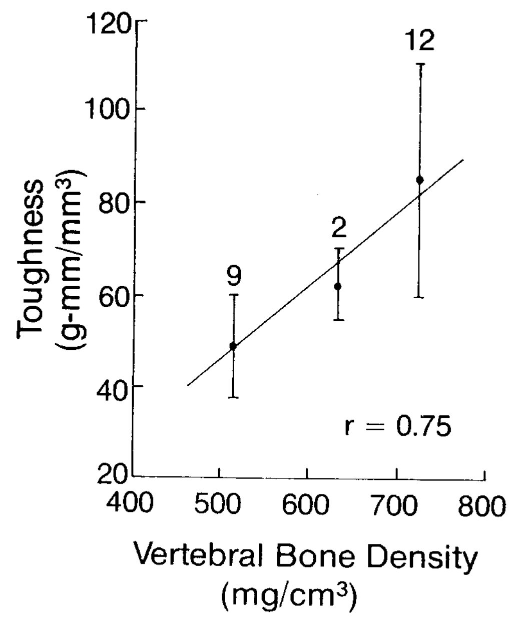

FIGURE 9.—*Relationship between vertebral energy-absorbing capacity (toughness) and bone density in bluegills aged 2, 9, and 12 months. Values are means ± 1 SD for each age.*

phasic response with age (Table 2). In channel catfish 6 months or older, bone strength increased with bone density (rupture $r = 0.77$, Fig. 6). The decline in bone density in older ages seemed responsible for the decline in vertebral strength. Human vertebral tissue exhibited similar bone density characteristics (Bartley et al. 1966; Rockoff et al. 1969). In 3-month-old channel catfish, bone density was higher than in either 15- or 24-month-old fish, but vertebral strength was less. Overall, relationships between mechanical properties and bone density in vertebrae were similar in channel catfish and brook trout. However, more research is needed to elucidate the relations among mechanical properties, density, and composition of bone in channel catfish younger than 6 months.

The correlation of organic matrix and of mineral aspects of bone composition with bone density may best explain the changes in the mechanical properties of bone observed in different ages of channel catfish. In early ages, high amounts of organic matrix, low hydroxyproline levels, and low density in bone tissue results in highly elastic, low-strength bone tissue. In intermediate ages, an optimal balance between biochemical composition (mineral : organic-matrix) and density occurs, resulting in peak bone strength (rupture and elastic limit values). In older ages, biochemical composition remains unchanged; however, bone-density reductions, probably due to senescence, weaken the structural integrity of bone as evidenced by reduced bone strength. The relationship between fish length, bone density, and bone strength for the five channel catfish ages is shown in Fig. 7. Adults showed no difference between sexes in mechanical properties of vertebra.

Bluegills

Vertebral strength declined and elasticity increased in bluegills between the ages of 2 and 12 months (Table 3). Vertebrae were stronger (increased rupture and elastic limit) and stiffer (increased ME) in 2-month-old than in either 9- or 12-month-old bluegills.

Hydroxyproline concentration within collagen as well as calcium and the mineral : organic-matrix ratio increased with bluegill age. Elevated mineral levels and minerals : organic-matrix ratios in 9- and 12-month-old bluegills suggested weakened structural integrity of vertebrae, which was demonstrated by the significant declines in vertebral strength observed in both ages. Increased hydroxyproline concentrations in collagen implied increased resiliency of bone organic matrix and were correlated with increased vertebral elasticity (ME $r = -0.72$, Fig. 8). The increase in hydroxyproline in backbone collagen may have strengthened the organic matrix and been partly responsible for the increased energy-absorbing capacity in 12-month-old fish.

Bone density increased between the ages of 2 and 12 months, as it did in early ages of brook trout and channel catfish. The increased bone density heightened the energy-absorbing capacity (toughness $r = 0.75$, Fig. 9).

Discussion

Mechanical properties of bone change with age in fish, and differ among species. Comparisons of the mechanical properties of bone in fish should be based on a single species. Within a species, size at age can vary (Hile 1936; LeCren 1951). In our study, fish length varied less than 10% within an age, but was highly correlated with size of vertebrae (brook trout $r = 0.98$; channel catfish $r = 0.98$; bluegill $r = 0.86$). Because the mechanical properties of bone incorporate vertebral size in the calculations, comparisons should be among fish of similar length, which have vertebrae of similar size.

The utility of developing a mechanical testing technique for bone lies in the evaluation of growth and development of bone tissue in fish. Bone composition in fish is altered by nutritional deficiencies (Halver et al. 1969; Wilson and Poe 1973), disease (Hoffman et al. 1962), and aquatic contaminants (Mayer et al. 1977; Mauck et al. 1978). Altered composition implies a potential alteration in integrity. If vertebral structural integrity is abnormal, individual vertebrae could be crushed or deformed by normal muscular contractions during swimming. Bengtsson (1979) reported that vertebral deformities in fish could be caused by hereditary factors, defective embryonic development, unsuitable water temperatures, low dissolved oxygen, radiation, dietary vitamin deficiencies, parasitic infection, electric current, and environmental contaminants. This mechanical-testing technique could be a diagnostic tool in studies of nutrition, disease, or aquatic contaminants. Applying mechanical testing techniques to these kinds of studies, investigators could relate changes in physiological or biochemical characteristics to structural integrity of a fish's skeleton.

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