Transactions of the American Fisheries Society 117:48–54, 1988

Bone Development in Creek Chub from a Stream Chronically Polluted with Heavy Metals

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Abstract.—Creek chub *Semotilus atromaculatus* from Rocky Fork, a metal-contaminated stream in north-central Ohio, and Clear Fork, a nearby uncontaminated stream, were studied to determine the effects of chronic heavy metal contamination from industrial and municipal sources on bone development. We examined bone development of fish from two contaminated sites in Rocky Fork and from four reference sites—three in Clear Fork and one in Rocky Fork—to evaluate variability among reference sites, and to determine if the clean-water headwater site on Rocky Fork was a suitable reference site for the two downstream contaminated sites. Collagen composition, density, strength, and energy-absorbing capacity of bone were significantly higher in fish from the headwater site on Clear Fork (upstream from an impoundment) than in fish at the other three reference sites. In bones of creek chub from sites in Rocky Fork where the water contained high total concentrations of chromium, copper, iron, nickel, and zinc, the collagen content of bone was lower but density and strength were higher than in bone of fish from the reference site. Some fish also had deformed vertebrae in the caudal peduncle region of the spinal column, which is a condition characteristic of neuromuscular overload of vertebrae. The high concentrations of chromium and zinc in fish from the contaminated sites probably interfered with collagen metabolism and altered the density and mechanical properties of bone. Creek chub at the contaminated sites seem to be tolerant of heavy metal contamination and may be adapting physiologically to the chronic metal stress in their environment.

Clear Fork and Rocky Fork are adjacent headwater tributaries of the Mohican River in north-central Ohio. Clear Fork is a relatively clean stream that flows through forested and agricultural lands and receives no major industrial discharges. Rocky Fork flows through the industrialized city of Mansfield and has received sewage and effluents with elevated concentrations of heavy metals since the mid-1930s (Ohio EPA 1983). A depauperate fish fauna in Rocky Fork within and downstream from the city of Mansfield was documented by both Reash (1984) and previous surveys by state agencies. Reash and Berra (1986) reported that water concentrations of total chromium, copper, iron, nickel, and zinc were higher in Rocky Fork below Mansfield than in either Clear Fork or at a reference site on Rocky Fork above Mansfield. They also reported significantly elevated whole-body concentrations of chromium and zinc in creek chub *Semotilus atromaculatus* from metal-contaminated sites. However, neither the chronic metal contamination nor elevated whole-body metals were associated with reduced mature egg production in these fish; fecundity of creek chub from metal-contaminated sites was similar to that of fish from reference sites.

Our objective was to evaluate bone development in creek chub from Clear Fork and Rocky Fork as a biological indicator of sublethal stress from exposure to heavy metals. Bone development was assessed by determining the quality (biochemical composition and density) and structural integrity (mechanical properties) of vertebrae. Previous studies of fish exposed in laboratory studies to environmental contaminants have shown that bone development can be adversely affected by lower contaminant concentrations or shorter exposure periods than those causing detectable changes in survival or growth (Hamilton et al. 1981a; Cleveland and Hamilton 1983; Hamilton et al. 1986). Characterization of bone development has also been used to evaluate the impact of environmental contaminants on fish (Mehrle et

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al. 1982; Baumann and Hamilton 1984; Buckley et al. 1985).

Methods

Forty-nine fish from previously studied sites (Reash 1984; Reash and Berra 1986) on Rocky Fork and Clear Fork were analyzed for bone development. Fish were collected with pulsed DC portable electrofishing gear during July 1982 and August 1983; 14 came from three reference sites in Clear Fork: 5 at site CF1, 3 at site CF2, and 6 at CF3; 35 came from three sites in Rocky Fork: 15 at reference site RF1, and 17 and 3 at contaminated sites RF3 and RF4, respectively (Figure 1; no fish were available from site RF2). Upstream movement of fish between RF1 and RF3 was unlikely, whereas downstream movement was possible, because a 2-m high industrial skimming dam and abundant stream debris blocked the entire stream width, but movement between sites RF3 and RF4 could have occurred. Clear Fork Reservoir prevented upstream movement from sites CF2 and CF3 to CF1. Detailed descriptions of sampling sites were given by Reash (1984). All fish were iced immediately upon collection and stored at −23°C until bone characteristics were determined. Fish from various sites were selected for characterization of bone development so that total length of selected animals was within the range 9–11 cm. This range was selected because it encompassed the majority of fish available for testing and because it met the criterion that there would be less than a 10% coefficient of variation (100·SD/mean) in total fish length between groups to be compared (Hamilton 1980). Comparison of bone mechanical properties of fish must be accomplished on fish of similar size and, concomitantly, on similar-sized vertebrae.

Fish were X-rayed before vertebrae were removed for bone analyses, and the radiographs were examined for vertebral and spinal deformities. Deformed vertebrae were not used in bone development determinations. Vertebrae 3 to 10 (counted from the skull) were individually dissected from each fish for testing of mechanical properties.

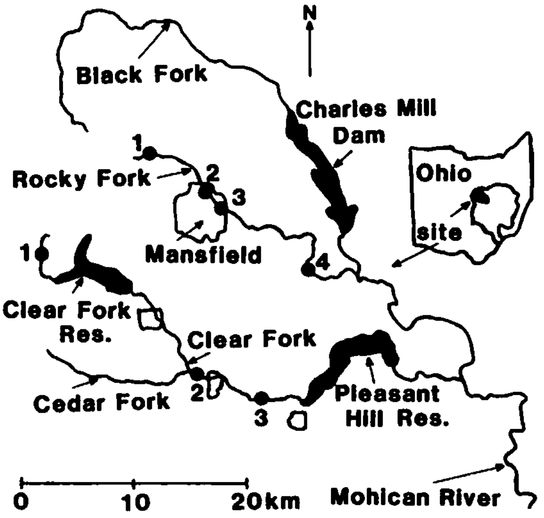

FIGURE 1.—Sampling sites for creek chub on Rocky Fork and Clear Fork, Richland County, Ohio.

Vertebrae were prepared and tested mechanically according to the method of Hamilton et al. (1981b). They were compression-tested by an Instron model 1132 testing machine with a constant strain rate of 5 mm/min to determine vertebral strength, elasticity, and energy-absorbing capacity (which was termed toughness). Measures of bone strength were elastic limit (the force level above which permanent structural damage occurs) and rupture (the force level causing failure of structural integrity). Elastic limit, i.e., stress at elastic limit, was measured as the force level at the proportional limit of the stress–strain curve and corresponded to the upper limit of the elastic region of the curve. Rupture, i.e., ultimate stress, was measured as the force level at the breaking peak or at pure plastic flow. Elasticity measures were strain (the amount of deformation incurred by bone tissue at failure) and modulus of elasticity (the index of bone stiffness or elasticity). Strain, i.e., ultimate strain, was measured as the amount of deformation incurred by bone at the failure point. Modulus of elasticity was measured as the slope of the elasticity region of the stress–strain curve. Toughness was measured as the total area under the stress–strain curve prior to the failure point and corresponded to the total work needed to break the bone.

After mechanical tests, we determined concentrations of collagen (Flanagan and Nichols 1962), hydroxyproline (Woessner 1961), proline (Troll and Lindsley 1955), calcium (Morin 1974), and phosphorus (Fiske and Subbarow 1925). Density of vertebrae was calculated as mean dry weight (mg) per mean volume (cm^3) of the eight vertebrae. Mean dry weight was the total weight of the vertebrae divided by the number of vertebrae in the pooled weight and mean volume was the average of measurements for individual vertebrae.

Site-specific effects of the reference sites on biochemical composition, density, and mechanical properties were determined by one-way analysis

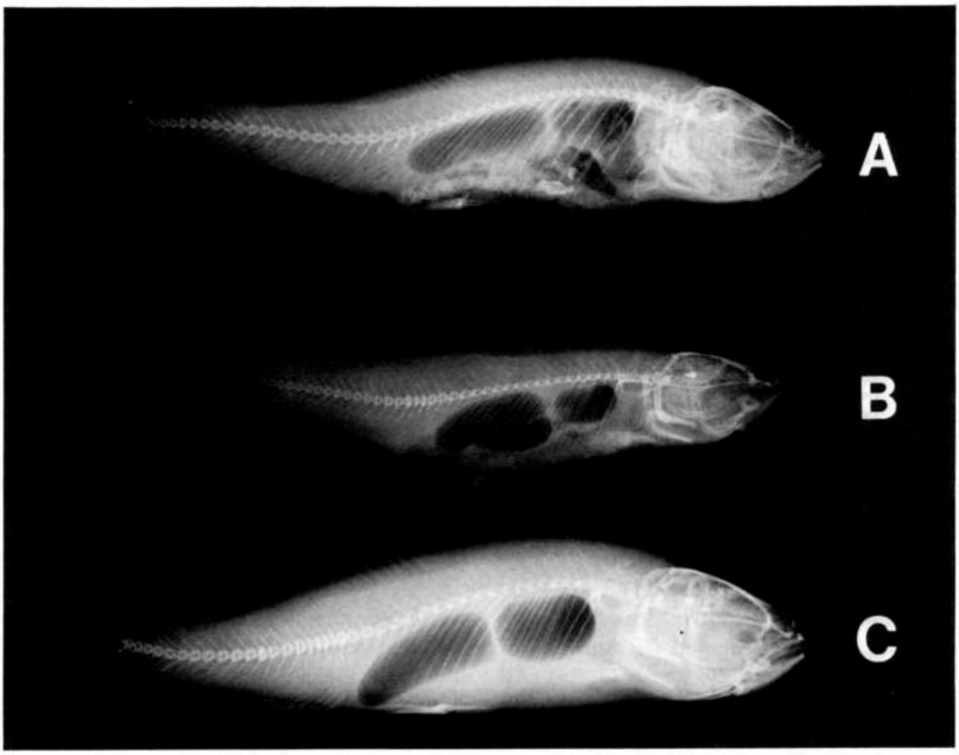

FIGURE 2.—Radiograph of creek chub from Rocky Fork, Ohio, with normal (A) and deformed (B, C) spinal columns.

of variance. Site means were compared by the least-significant-difference, multiple-mean comparison test (Snedecor and Cochran 1967). Differences between combined reference sites and combined contaminated sites were determined by *t*-test.

Results

The three clean-water sites in Clear Fork (CF1–CF3) and one site in Rocky Fork (RF1) were potential reference locations for comparison of fish from uncontaminated sites with those from contaminated sites RF3 and RF4. The sizes of the fish from the four reference sites were different, but the coefficient of variation for all fish sampled at the four reference sites was 1.5%, thus indicating that fish size and, concomitantly, vertebral size were similar at the different locations. This amount of variation is well within the suggested upper limit of 10% for comparisons of density and mechanical properties of bone in fish of similar size (Hamilton 1980). In previous studies, differences in density and mechanical properties were evaluated with one-way analysis of covariance in which fish length was used as the covariable. However, in the present study, there was no difference in the statistical results of the analysis of variance and the analysis of covariance. The small variation in length of fish among reference sites explains the lack of difference in statistical results of these two tests.

Radiographs used in the evaluation of bone development showed no vertebral deformities in creek chub from Clear Fork (CF1–CF3) but deformed vertebrae occurred in 1 of 15 fish from RF1, 4 of 17 from RF3, and 2 of 3 from RF4. Deformed vertebrae were in the posterior spinal column near the caudal peduncle and generally involved vertebrae 18–26 posterior to the skull (Figure 2).

Bone characteristics of fish from site CF1 were significantly different from those of fish from the other three reference sites; bone collagen concentration, density, and mechanical property values were all higher than in fish from the other three reference sites (Table 1). Vertebrae of fish from CF1 were stronger (increased rupture and elastic limit), stiffer (increased modulus of elasticity), and absorbed more energy before failure (increased

TABLE 1.—Means (±SD) of bone development characteristics and of size of creek chub from four reference sites in two streams, Clear Fork and Rocky Fork, Ohio. Values in the same row with letters in common are not significantly different from each other ($P \leq 0.05$).

	Location			
	Clear Fork			Rocky Fork, site 1
Characteristic	Site 1	Site 2	Site 3	
Biochemical composition				
Collagen (mg/g bone)	303±8 z	263±7 y	286±9 x	291±12 x
Proline (mg/g bone)	37.5±1.4 z	36.6±1.6 z	36.4±2.1 z	38.6±4.2 z
Hydroxyproline (mg/g bone)	24.3±1.4 z	24.8±1.3 z	24.7±0.9 z	25.2±1.7 z
Calcium : phosphorus ratio	1.9±0 z	1.8±0.1 z	1.9±0.1 z	1.9±0.1 z
Ca+P : collagen	1.1±0.1 z	1.2±0.1 zy	1.2±0 zy	1.2±0.1 y
Density (mg/cm^3)	466±32 z	381±32 y	370±30 y	382±33 y
Mechanical properties				
Rupture (g-force/mm^2)	1,420±367 z	823±82 y	844±121 y	919±97 y
Elastic limit (g-force/mm^2)	1,131±318 z	654±81 y	651±94 y	668±74 y
% strain	10.4±2.3 z	10.3±1.4 z	9.1±0.9 z	10.8±2.5 z
Modulus of elasticity (kg-force/mm^2)	21.6±7.4 z	11.7±0.5 y	13.9±1.9 y	14.3±2.4 y
Toughness (g-mm/mm^3)	94±36 z	54±13 y	49±13 y	64±20 y
Fish size				
Standard length (cm)	10.8±0.8 z	9.9±0.5 y	9.1±0.3 x	9.9±0.6 y
Weight (g)	20.4±4.8 z	17.0±2.5 zy	14.7±1.9 y	16.4±2.8 y
Number of fish sampled	5	3	6	15

toughness) than vertebrae of creek chub from the other three reference sites.

Except for collagen, bone characteristics were not significantly different between sites RF1, CF2, and CF3. Moreover, there were no significant differences in bone characteristics between fish from contaminated sites RF3 and RF4. Consequently, the observations for these three reference sites were combined for comparison with the combined contaminated sites. Bone development of fish from the combined contaminated sites was significantly different from that of fish from the combined reference sites (Table 2). Collagen metabolism in bone tissue was significantly altered in creek chub from the contaminated sites compared with those from the reference sites because collagen, hydroxyproline (a key crosslinking amino acid in collagen), and its precursor proline were significantly reduced. Mineralization, as measured by calcium and phosphorus, was unaltered, however. Consequently, the ratio of minerals to collagen was significantly higher in creek chub from the contaminated sites than in fish from the reference sites. The reduction in concentrations of collagen, hydroxyproline, and proline in bone ranged from 3 to 7%. Bone density was significantly increased about 11% in fish from the contaminated sites compared to those from the reference sites. Bone strength (rupture), elasticity (strain and modulus of elasticity), and toughness in fish from the contaminated sites were significantly higher than in fish from the reference sites. There was a much greater change in the mechanical properties of bone than in biochemical and density characteristics; in bone from the contaminated sites, rupture was increased 20%, strain 19%, modulus of elasticity 6%, and toughness 46% over bone from the reference fish.

TABLE 2.—Means (±SD) of bone development characteristics and of size of creek chub from three combined reference sites and two combined contaminated sites in two streams, Clear Fork (CF) and Rocky Fork (RF), Ohio. Values in the same row with letters in common are not significantly different from each other ($P \leq 0.05$).

	Sites	
Characteristic	Reference (CF2, CF3, RF1)	Contaminated (RF3, RF4)
Biochemical composition		
Collagen (mg/g bone)	286±14 z	275±15 y
Proline (mg/g bone)	37.8±3.6 z	35.0±1.1 y
Hydroxyproline (mg/g bone)	25.0±1.5 z	24.2±1.1 y
Calcium : phosphorus ratio	1.9±0.1 z	1.9±0.1 z
Ca+P : collagen	1.2±0.1 z	1.3±0.1 y
Density (mg/cm^3)	379±31 z	420±63 y
Mechanical properties		
Rupture (g-force/mm^2)	888±106 z	1,069±301 y
Elastic limit (g-force/mm^2)	662±77 z	736±264 z
% strain	10.3±2.2 z	12.3±2.2 y
Modulus of elasticity (kg-force/mm^2)	13.9±2.3 z	14.7±4.1 z
Toughness (g-mm/mm^3)	59±18 z	83±27 y
Fish size		
Standard length (cm)	9.7±0.6 z	9.7±0.8 z
Weight (g)	16.1±2.6 z	19.1±5.0 y
Number of fish sampled	24	20

Discussion

Comparison of Reference Sites

The unique separation of bone mechanical properties of fish from site CF1 from those of fish from CF2, CF3, and RF1 was unexpected. Sites CF2 and CF3 were 8.5 km apart, but were not separated by a barrier, and the creek chub at the two sites may have represented a single population. The bone characteristics of fish from RF1 were similar to those of fish from CF2 and CF3. Bone development in fish of the same species and age, reared under similar environmental conditions (water quality, temperature, watershed geology, diet), should have similar physiological development, as seen at three of the reference sites. No differences in water quality and temperature characteristics were recorded among the four reference sites (Reash 1984). Although no information was collected on diet, it may have been a factor in the differences observed in fish from CF1.

The unique bone development in fish from CF1 may have been a result of physical adaptation to habitat stress. The mean current velocity at CF1 and RF1 was similar (0.18 and 0.22 m/s, respectively) but the upper limit at CF1 was 0.5 compared to 0.3 at RF1 (Reash 1984). The mean volume of flow at CF1 was 0.09 m^3/s with an upper limit of 0.35, compared with a mean value of 0.05 and upper limit of 0.08 at RF1. Site CF1 was also more variable than other reference sites in measures of alkalinity, hardness, dissolved oxygen, and conductivity (Reash 1984). The lower variability of physicochemical conditions at RF1 was due to its origin from a continuous flowing spring; likewise, conditions at CF2 and CF3 were moderated by continuous releases from Clear Fork Reservoir. Variability of physicochemical conditions is greater in headwaters than in environmentally stable middle and downstream locations in streams and rivers (Hynes 1970; Horwitz 1978). Fish from intermittent headwaters are more tolerant of variable physicochemical conditions such as dissolved oxygen, temperature, and pH than are fish from the river mainstream (Matthews and Styron 1981). Moreover, Matthews and Styron (1981) reported that intraspecies differences in physiological tolerances among populations in a single watershed could help isolate populations (which, in our study, was facilitated by the presence of Clear Fork Reservoir), an initial step in the speciation process. The fish population at CF2 and CF3, although separated from Rocky Fork populations by Pleasant Hill Reservoir, seemingly were not different physiologically (as judged by bone development analyses) from fish inhabiting RF1. Nevertheless, mechanical property tests clearly demonstrated that fish at CF1 had bone structural integrity that was substantially stronger and tougher than observed in fish from the other three reference sites.

Heavy Metals in Rocky Fork

The lower reaches of Rocky Fork have been contaminated by high concentrations of heavy metals and sewage since the mid-1930s (Ohio EPA 1983). Reash and Berra (1986) reported elevated but variable total metal concentrations of chromium, nickel, and zinc in water at polluted sites on Rocky Fork, suggesting that fish were exposed to intermittently high concentrations. These elevated concentrations of metals perhaps exerted chronic toxicity effects on stream fishes that remained. Sensitive fish species were absent in polluted sites on Rocky Fork, and species tolerant of contaminated habitats, e.g., creek chub and white sucker *Catostomus commersoni* (Leonard and Orth 1986), were the only species collected at RF3 and RF4 (Reash 1984). However, Reash and Berra (1986) suggested that the toxicity of metals was partly mitigated by complexation, because the fecundity of the creek chub inhabiting Rocky Fork within and downstream of Mansfield was not altered.

Effects of Heavy Metals on Bone Development

Deformities of vertebrae in the caudal peduncle region of creek chub at RF3 and RF4 were similar to those reported by Bengtsson et al. (1985), who suggested that exposure to cadmium, lead, or zinc could cause the neuromuscular complex to overload vertebrae through elevated muscle tone and tetanic coma. This would result in skeletal deformities in the caudal peduncle, where the propulsive force of the swimming musculature is centered (Webb 1984). Similar deformities have been reported by Bellanca and Bailey (1977) for adult fish exposed to chlorinated wastewater effluents in the James River estuary, Virginia. Deformities of fish from the RF4 site may have been related to the chlorinated effluents from the Mansfield wastewater treatment plant although total chlorine residues measured by Reash (1984) below the plant were low (mean, 0.1 mg/L; range, 0–0.6 mg/L).

Reash (1984) reported that creek chub, white sucker, and blacknose dace *Rhinichthys atratulus* accounted for most fish with external deformities in Rocky Fork. Leonard and Orth (1986) reported that these same three species had the highest incidence of anomalies (i.e., bloating and fin and skeletal anomalies) in West Virginia streams having a wide range of stream degradation. In the present study, the frequency of deformities in creek chub ranged from 0.23% (21 of 6,483) to 0.32% (4 of 1,527) for clean-water sites on Clear Fork and Rocky Fork, and was 0.75% (9 of 1,209) for contaminated RF3 (Reash 1984). Bengtsson et al. (1985) observed a similar increase in the frequency of vertebral deformities in fish collected near several metal smelters compared with those collected at unpolluted locations. Although selective pressures would be expected to eliminate severely deformed fish from any population, frequencies as high as 20% have been reported for severely deformed white crappie *Pomoxis annularis* in Lake Decatur, Illinois (Baumann and Hamilton 1984).

The altered collagen metabolism in creek chub from the contaminated sites suggested that metabolic interference from zinc and (to a lesser extent) nickel might have altered the formation of mature collagen and its amino acid composition. Oikarinen et al. (1984) reported that zinc, copper, and mercury inhibited the calcium-dependent conversion of procollagen to collagen in the presence of calcium and that nickel, lead, cobalt, and manganese inhibited this conversion in the absence of calcium. Moreover, nickel has been reported to accumulate in bone of white suckers exposed to heavy metals in streams (Ney and Van Hassel 1983); thus its increased presence in bone could have made it a major contributor to the reduction in collagen metabolism observed in our study. Chromium also has been reported to accumulate in the opercular bone (and probably other bony tissues) of rainbow trout *Salmo gairdneri* exposed for 2 years to 0.25 μg/L (Buhler et al. 1977) and may have influenced bone development.

The presence, in chronically polluted sites in Rocky Fork, of creek chub with elevated body burdens of chromium and zinc but normal fecundity (Reash and Berra 1986), as well as with elevated bone density and mechanical properties, may indicate that this tolerant species is adapting to its contaminated habitat. Our results may be analogous to those of McFarlane and Franzin (1978), who reported that white suckers in a metal-contaminated lake showed biological and life history features suggesting that the species was adapting to or compensating for chronic pollution. Elevated bone density and strength probably reduce the potential overload of vertebrae from heavy-metal-induced neuromusculature contractions. Spinal deformities in fish reduce swimming performance (Bengtsson 1974; Weis and Weis 1976) and, hence, reduce the fitness of deformed animals to compete with unaffected fish. Elevated density and strength of bone in creek chub may confer a selective advantage in competition for food, escape from predators, or defense of spawning areas.

Acknowledgments

We thank Jim Dwyer, Paul Heine, Sharna King, and Dan Witt for assistance in the analysis of bone characteristics, two anonymous reviewers for their critical reviews of the manuscript, and Kathern Schilling for preparing several revisions of the typescript. This study was part of a Master's thesis by Rob J. Reash and was supported by grants from the U.S. Army Corps of Engineers (contract DACW69-83-M-0698) and the Sigma Xi Society.

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Received April 3, 1987
Accepted March 7, 1988