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# A Digenean Metacercaria (*Apophallus* sp.) and a Myxozoan (*Myxobolus* sp.) Associated with Vertebral Deformities in Cyprinid Fishes from the Willamette River, Oregon

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*Abstract.*—A high prevalence of vertebral deformities has been observed in various fishes, especially cyprinids, from certain regions of the Willamette River for many years. One proposed source of these deformities is exposure to toxicants. Histological evaluation of affected chiselmouth *Acrocheilus alutaceus* revealed that all lesions associated with vertebrae were associated with metacercariae of digenean trematodes. Approximately half of the northern pikeminnow *Ptychocheilus oregonensis* had infections in which metacercariae were associated with these lesions. Metacercariae were also associated with vertebral lesions in three of four affected peamouth *Mylocheilus caurinus*. Many metacercariae that were present within the vertebral bodies were associated with bony dysplasia and bony proliferation in all three species. We also evaluated the association of the metacercariae with the vertebral deformities, using intact fish that had been cleared with trypsin. Fish from the affected regions had a much higher prevalence of metacercariae and deformities and a greater abundance of metacercariae than those in the reference site. Chiselmouths had more deformities and metacercariae than northern pikeminnow. In all fish species, 77% of deformities were directly associated with metacercariae; in chiselmouths, about 95% of the deformities exhibited this relationship. Two types of metacercariae were identified in affected fish: *Apophallus* sp. (Heterophyidae) and a neascus type (Strigeidida). The *Apophallus* sp. appeared to be more closely associated with the skeleton deformities. A *Myxobolus* sp. morphologically similar to *M. cyprini* was also associated with the vertebral lesions in about 50% of the northern pikeminnow and 5% of the chiselmouths. Intact plasmodia were found in somatic muscle, and lesions containing free spores were often located at bone surfaces. This survey demonstrates that metacercariae (probably *Apophallus* sp.) and a *Myxobolus* sp. are major causes of the vertebral deformities seen in cyprinid fishes from certain regions of the Willamette River.

Certain regions of the Willamette River in Oregon are considered to have been impacted by anthropogenic contamination, and the U.S. Environmental Protection Agency has declared Portland Harbor a Superfund site (Waite and Carpenter 2000). The Oregon Department of Environmental Quality (ODEQ) has reported a high prevalence of vertebral anomalies in various fish species from

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this river. The ODEQ studies found that 1–74% of northern pikeminnow *Ptychocheilus oregonensis* in the Willamette River had vertebral anomalies, the greatest prevalence being found at river mile (RM) 27–55, an area known as Newberg Pool (Ellis et al. 1997). The prevalence of deformities generally decreased with distance upstream and downstream of Newberg Pool. In a subsequent survey by Markle et al. (2002) no differences in the patterns of caudal deformities in Willamette River fishes were observed between RM 29 and 71, whereas precaudal deformities were substantially more numerous around the Newberg Pool area (RM 45–50). Most of the 14 fish species in which they reported vertebral deformities were cyprinids, these deformities being most prevalent in chiselmouth *Arcocheilus alutaceus*.

Skeletal deformities in fishes can be caused by various contaminants, including cadmium, lead, zinc, and organophosphate pesticides (Bengtsson 1974; Bengtsson et al. 1975, 1985; Hiraoka and Okuda 1983). Therefore, vertebral deformities in fish have been suggested to serve as bioindicators of pollution (Bengtsson 1979; Lemly 1997). However, other causes of vertebral deformities are not directly related to chemicals, such as low pH (Frojnar 1977), warmer temperature (Kwain 1975), and nutritional deficiencies (Mehrle and Meyer 1975, 1982; Mauck et al. 1978; Bengtsson 1979; Walton et al. 1984; Akiyama et al. 1986). Moreover, several infectious agents can cause vertebral deformities in fish, including viruses (LaPatra et al. 2001), bacteria (Conrad and DeCrew 1967; Kent et al. 1989), and a wide variety of parasites—particularly myxozoans and metacercariae of digenean trematodes (e.g., Baturo 1980; Bucke and Andrews 1985; Hedrick et al. 1998; Matthews et al. 2001).

A multidisciplinary effort was undertaken by toxicologists, fisheries biologists, and pathologists to elucidate the cause of these deformities in fishes from the Willamette River, including investigations into potential chemical or infectious causes. Here we report the prevalence, associated pathological changes, and description of parasites from northern pikeminnow, chiselmouths, and peamouths from the Willamette River, Oregon.

## Methods

*Samples and specimen processing.*—Underyearling and yearling northern pikeminnow, chiselmouths, and peamouths were collected from the Willamette River near the affected zones: Ash Island, Newberg Pool (RM 51.5), and Wheatland Ferry (RM 72). The reference site was Kiger Island, which is near Corvallis (RM 135) on the Willamette River. All fish were collected from 20 to 29 July 2002. Fish were collected by beach seine, killed with an overdose of Finquel (tricaine methanesulfonate; Argent Chemical Laboratories, Redmond, Washington) and preserved in 10% buffered formalin. Preserved whole fish in formalin were radiographed with a Faxitron MX-20 radiography unit, and the images were evaluated for the presence of vertebral deformities as described by Markle et al. (2002).

Preserved fish were decalcified before processing for histology. Tissues were placed in Cal-Ex II solution (Fisher Scientific, Pittsburgh, Pennsylvania) for 24–48 h. Samples were then rinsed in running tap water for 2 h, after which they were returned to 10% buffered formalin for 24 h. Tissues were embedded, and sagittal sections prepared by standard histological techniques were stained with hematoxylin and eosin. Larger fish were divided into thirds in cross sections before processing. Three 6-μm-thick sections were prepared from each block at approximately 50-μm intervals.

To allow for better correlation with the presence of metacercariae associated with vertebral changes, we also examined fish in whole mounts in which fish were first cleared with trypsin and stained with alcian blue and alizarin red S (Dingerkus and Uhler 1977; Potthoff 1984). Cleared fish were placed in a Petri dish and covered with glycerin; the entire fish was then examined at 25× or 50× magnification. Total numbers of metacercariae and the presence and absence of the parasites directly associated with vertebral deformities were recorded. Morphological information from the parasites was obtained by using wet mount preparations, and all measurements of metacercariae and myxozoan spores were made with a SPOT camera and computer program (SPOT versions 3.5.5. for Windows; SPOT Diagnostic Instruments, Sterling Heights, Michigan).

For molecular analysis of the myxozoan, muscle tissue determined by microscopy to contain *Myxobolus* sp. spores was frozen or placed in 95% ethanol. To extract DNA, we used the DNeasy Kit (QIAGEN Inc., Valencia, California). A region of the small subunit (SSU) ribosomal DNA (rDNA) was amplified by means of the polymerase chain reaction (PCR) with primers (18E and Myxgen2r) and conditions as described by Kent et al. (2000). Using the above PCR primers and the methods of Whipps et al. (2003), we obtained a sequence ap-

TABLE 1.—Histological evaluation of the prevalence and association of parasites with lesions compared with vertebral deformities identified by radiograph (X-ray) analysis in chiselmouths (CHM), northern pikeminnow (NPM), and peamouths (PEAM) from the Willamette River. Chiselmouths were identified as underyearlings, peamouths as yearlings, and northern pikeminnow as either underyearlings (−1) or fish more than 1 year old (+1). The term ''lesions'' indicates parasites associated with vertebral lesions. Note that fewer lesions were detected by histology in fish that were positive for deformities by radiography because vertebral deformities were not always located by the former method.

| | | | Metacercariae | | *Myxobolus* | |
|---|---|---|---|---|---|---|
| Fish | X-ray | No. | Prevalence (%) | Lesions (%) | Prevalence (%) | Lesions (%) |
| NPM (−1) | Positive | 28 | 12/28 (43) | 12/24 (50) | 9/28 (32) | 8/24 (33) |
| NPM (−1) | Negative | 18 | 4/18 (22) | 2/3 (0) | 5/18 (25) | 0/3 (0) |
| NPM (−1) | Combined | 46 | 16/46 (35) | 14/27 (37) | 14/46 (30) | 8/27 (30) |
| NPM (+1) | Positive | 21 | 16/21 (76) | 3/17 (18) | 18/21 (86) | 13/17 (76) |
| NPM (+1) | Negative | 6 | 1/6 (16) | 0/0 (0) | 2/6 (33) | 0/0 (0) |
| NPM (+1) | Combined | 27 | 17/27 (63) | 0/0 (0) | 20/27 (74) | 0/0 (0) |
| CHM | Positive | 21 | 21/21 (100) | 21/21 (100) | 2/21 (9) | 0/12 (0) |
| CHM | Negative | 5 | 4/5 (80) | 0/0 (0) | 1/5 (20) | 0/0 (0) |
| CHM | Combined | 26 | 25/26 (96) | 0/0 (0) | 2/26 (8) | 0/0 (0) |
| PEAM | Positive | 4 | 3/4 (75) | 3/4 (75) | 0/4 (0) | 0/4 (0) |
| PEAM | Negative | 4 | 1/4 (25) | 0/0 (0) | 0/4 (0) | 0/0 (0) |
| PEAM | Combined | 8 | 4/8 (50) | 0/0 (0) | 0/8 (0) | 0/0 (0) |

proximately 850 base pairs (bp) long. To complete the sequence, the 3′ region of the SSU rDNA was amplified and sequenced with primers Mcyp1F (5′-GTCAGTTTGTAGTCTGCG-3′) and 18R of Whipps et al. (2003). Combining overlapping sequences yielded a 1911-bp sequence, which we deposited in GenBank (accession number AY591531).

The sequences used for alignment and subsequent phylogenetic analyses were those of Molnár et al. (2002). Additional sequences were selected on the basis of GenBank searches using the basic loc alignment search tool (BLAST; Altschul et al. 1990). These sequences (and their GenBank accession numbers) were as follows: outgroup *M. lentisuturalis* (AY119688), *M. bibullatus* (AF378336), *M. bartai* (AF186835), *M. pseudodispar* from rudd *Scardinius erythrophthalmus* (AF380142), *M. pseudodispar* from roach *Rutilus rutilus* (AF380145), *M. pseudodispar* from bream *Abramis brama* (AF380144), *M. pseudodispar* from silver bream *Blicca bjoerkna* (as known as *Abramis bjoerkna*; AF380143), *M. cyprini* (AF380140), *M. musculi* (AF380141), and Triactinomyxon sp. (AY162270). Sequences were aligned by using ClustalX (Thompson et al. 1997) and analyzed with PAUP*4.0b1 (Swofford 1998). For parsimony analysis we used a heuristic search algorithm with 50 random sequence additions. Bootstrap confidence values were calculated with 1,000 repetitions.

*Statistics*.—All statistical analyses were performed at $\alpha = 0.05$ with Statgraphics Plus software (Manugistics 2000). For cleared fish we used a multifactor analysis of variance (ANOVA) to determine the effects of the number of metacercariae, fish species, fish size, and river location on the total number of deformities per fish. Peamouths were excluded from this analysis because of small sample size. We also used linear regression to illustrate the relationship between the number of deformities and the number of metacercariae. Examining this relationship in northern pikeminnow and chiselmouths showed that the slopes and intercepts for each species were not different, so that we pooled data from both species. Histological data are presented as a cross-tabulation for presence or absence of metacercariae and *Myxobolus* sp. in lesions; independence of parasite–lesion relationships was evaluated with a chi-square test.

## Results

### *Histology*

Histopathologic evaluations of fish from an affected site (Ash Island) detected lesions (vertebral deformities and associated chronic inflammation) in most of the fish that had vertebral deformities as determined by radiography (Table 1). A total of 8% (3/38) of fish from this site that lacked vertebral deformities (by radiography) exhibited these histological changes. Many of the lesions were associated with either metacercariae or myxozoans, and both parasites were more prevalent in fish with vertebral deformities than in those without such deformities as determined by radiographic analysis (Table 1). Differences were seen among host species: Almost all the lesions in chiselmouths and

peamouths were associated with the metacercariae, whereas in northern pikeminnow 50% of lesions were associated with *Myxobolus* sp. and 36% were associated with metacercariae.

Metacercariae and myxozoan spores were observed in or near vertebrae, spines, and the pectoral girdles of many fish for all three host species (Figure 1). Metacercariae were observed either within bone or appressed directly to the surfaces of vertebrae in which the lesion could be located. The metacercariae were associated with prominent dysplastic and proliferative responses of adjacent bone, often resulting in complete encapsulation of the parasite (Figure 1). Metacercariae observed to be directly associated with vertebral spines were either encapsulated by new bone or present within spines. In both cases, the bony capsule was indistinguishable from bone of normal structures. Examination of radiographs revealed clear regions within dense bone that corresponded to the presence of the parasite (Figure 2).

A histozoic *Myxobolus* sp. was observed in northern pikeminnow and chiselmouths but not in peamouths (Table 1). The myxozoan occurred as intact plasmodia between muscle fibers, often adjacent to vertebral spines. Individual spores were found within areas of chronic inflammation adjacent to vertebrae and the spines of some fish (Figure 3). The inflammatory response consisted of melanin-laden macrophages and associated fibroplasia.

Adding data for the chiselmouths and northern pikeminnow from the reference site (Kiger Island) to the data for Ash Island demonstrated that metacercariae were associated with 53% (32/60) of fish with histologically verifiable vertebral lesions (Table 2). Furthermore, *Myxobolus* sp. was associated with 36% (22/60) of fish with histologically verifiable lesions. Using a chi-square test for cross-tabulation for the presence or absence of metacercariae and *Myxobolus* sp. in lesions observed by histology indicated that a lesion associated with a metacercaria was very seldom associated with *Myxobolus* sp. and conversely that a lesion associated with *Myxobolus* sp. very seldom had metacercariae; only 2 of 63 lesions had both (Table 3).

### *Cleared Whole Mounts*

Radiographic analysis provides an entire view of the vertebrae of small fish, whereas histology is essentially a two-dimensional technique, revealing only relatively small portions of the skeleton in each slide. We were concerned that some deformities seen by radiography were not observed by histology because of the difficulty of locating vertebrae with lesions (Table 1). Therefore, we examined fish in cleared whole mounts in an attempt to better determine the association of the metacercariae with the vertebral deformities. Both metacercariae and deformities were readily visible with this technique. Large aggregates of *Myxobolus* spores in plasmodia were observed, but free spores associated with inflammation and vertebral deformities were not. Vertebral deformities (including lordosis, the absence of vertebral spines, additional vertebral spines, and bone proliferation) and their association with metacercariae were further elucidated by this method (Figure 4). Multiple, ovoid, pleomorphic, dense, alizarin red–positive bodies (mineralized foci in granulomatous inflammation) were frequently associated with parasites in the surrounding musculature (Figure 4G). In most of the vertebral deformities evaluated by this method, metacercariae were directly associated with the bone, particularly in chiselmouths (Table 4).

Chiselmouths had more than 7.5 times the abundance (mean number of parasites/individual, including uninfected individuals) and about 2.5 times the prevalence of metacercariae as northern pikeminnow in the affected sites (Table 4). A similar pattern was observed between these two species in the Corvallis reference site. Intraspecies comparisons revealed that both species from the affected sites had about 3 times the deformity prevalence, 2–3 times the prevalence of infection by metacercariae, and 4–6 times the abundance of metacercariae compared with those fish at the reference site in Corvallis. Most importantly, vertebral deformities were consistently associated with the metacercariae. This phenomenon was particularly evident in chiselmouths, which had the highest prevalence and abundance of infection by the metacercariae. Combined data regarding vertebral deformities from both host species from all sites indicated that 44 of 57 (77%) of the lesions were directly associated with visible metacercariae. Multifactor analysis demonstrated that the number of vertebral deformities was related to the number of metacercariae but not to the fish species, fish size, or location in river. Hence, we could appropriately combine fish species, sizes, and areas in our other analyses. Regression analysis comparing the number of metacercariae with the number of lesions found in the cleared fish data (combining northern pikeminnow and chiselmouths) showed that the number of lesions increased linearly with metacercaria abundance ($Y = 0.30 +$

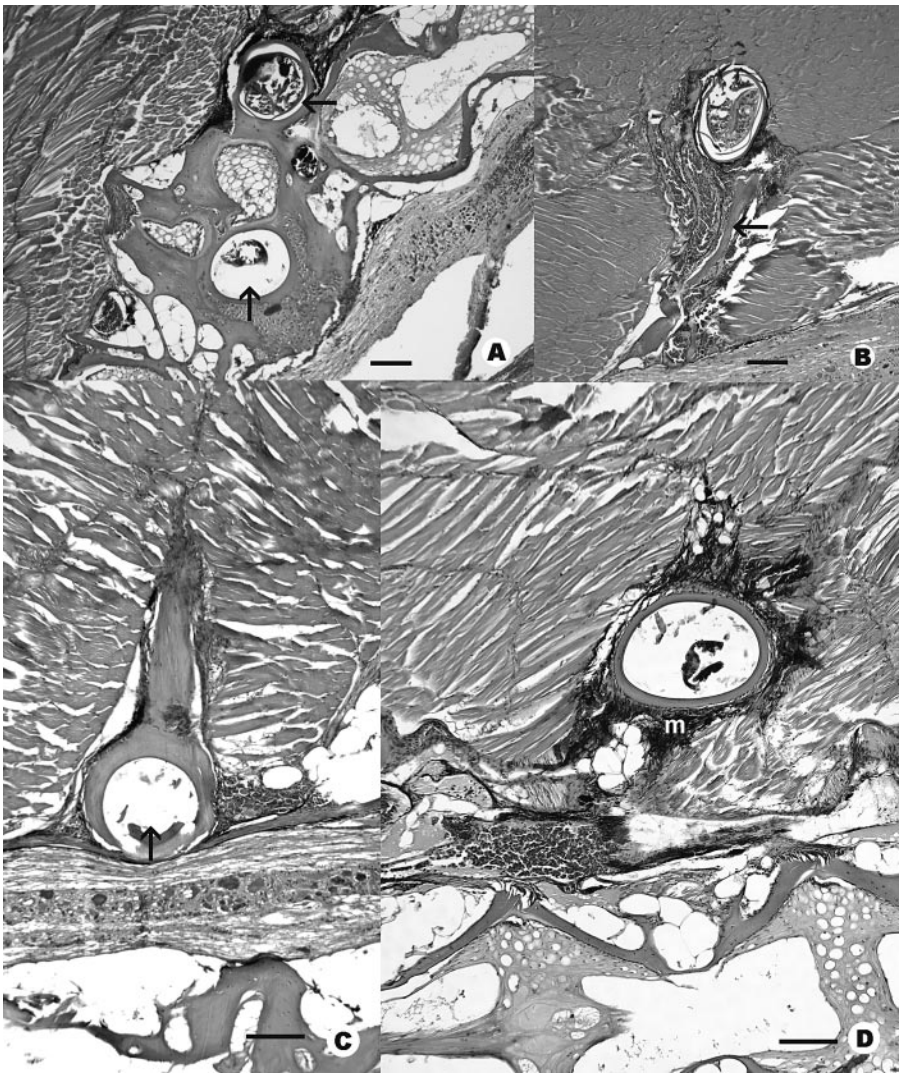


FIGURE 1.—Histological sections of metacercariae in (**A, B,** and **D**) northern pikeminnow and **(C)** peamouths. Hematoxylin and eosin stain was used; bars = 100 μm. Panel (A) shows encysted metacercariae (arrows) in a vertebra associated with bone and cartilage proliferation, panel (B) metacercaria associated with chronic inflammation and a deformed dorsal spine (arrow), panel (C) a bony cyst with a spherical space (arrow) suggesting the location of metacercariae in a neural spine at the base of a vertebra, and panel (D) bony cysts in the spine with melanized host reaction (m).

0.13*X*, adjusted $r^2 = 0.23$, $P < 0.0001$). The data for each species were combined in the analysis because regressions of each species separately did not differ significantly in intercepts ($P = 0.30$) or slopes ($P = 0.14$).

Aggregates of myxozoan spores in the somatic muscle were seen in several northern pikeminnow from Ash Island but not in fish from the Corvallis reference site (Table 4). The spores were not observed in the other samples and were not directly associated with lesions, as determined using the cleared whole fish method.

*Parasite Identifications*

Wet-mount preparations of metacercariae removed from cysts near vertebrae revealed a heterophyid metacercaria consistent with *Apophallus* sp. (Figure 5A). Distinctive features of the metacercaria identified as *Apophallus* sp. included spiny tegument, oblique testes, relatively small acetab-

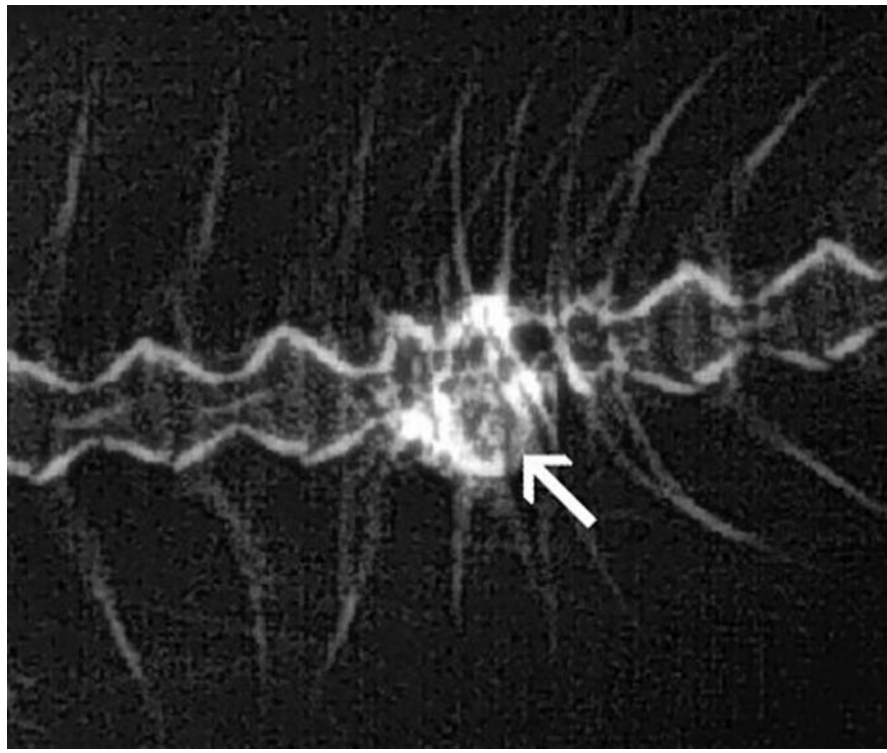

FIGURE 2.—Radiograph of a vertebral deformity in a chiselmouth. The arrow indicates a region suggestive of metacercarial infection; the dense region represents bone proliferation with a lucent center corresponding to an encysted worm.

ulum and oral sucker, short prepharynx, small pharynx, and a very long esophagus, which resulted in division of the caecum in the posterior region of the worm (Schell 1985). Metacercariae of the *Apophallus* sp. measured about 265 μm ($n$ = 2). Strigeid metacercariae (neascus type) with obvious calcareous corpuscles were found in the muscle and fins within large black, spherical cysts; these metacercariae measured about 200 μm ($n$ = 3; Figure 5B).

The myxozoan spores observed in muscle tissue of both northern pikeminnow and chilsemouths were morphologically consistent with those of the genus *Myxobolus*. Spores most closely resembled those of *M. cyprini*, which has polymorphic spores that infect muscle tissue of cyprinid fishes (Molnár et al. 2002). Some spores were symmetrical with apical polar capsules; others had unequal valves and subapical polar capsules (Figure 6). Spore measurements were as follows ($n$ = 30, values in μm) length = 11.0 (range, 10.1–12.6), width = 7.2 (6.7–7.7), large polar capsule length = 6.6 (5.5–7.1), and small polar capsule length = 5.3 (4.0–5.9).

We sequenced 1,911 bp of the SSU rDNA of the *Myxobolus* sp. from northern pikeminnow and

TABLE 2.—Cross-tabulation of the relationships between detection of vertebral deformities via radiographs and histological lesions in chiselmouths, northern pikeminnow, and peamouths from Ash and Kiger islands; neg. = negative, pos. = positive.

| X-ray deformity | Histological lesions | | Metacercariae with lesions | | *Myxobolus* with lesions | | Total |
|---|---|---|---|---|---|---|---|
| | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. | |
| Neg. | 35 | 3 | 36 | 2 | 37 | 1 | 38 |
| Pos. | 20 | 60 | 48 | 32 | 58 | 22 | 80 |
| Total | 55 | 63 | 84 | 34 | 95 | 23 | 118 |

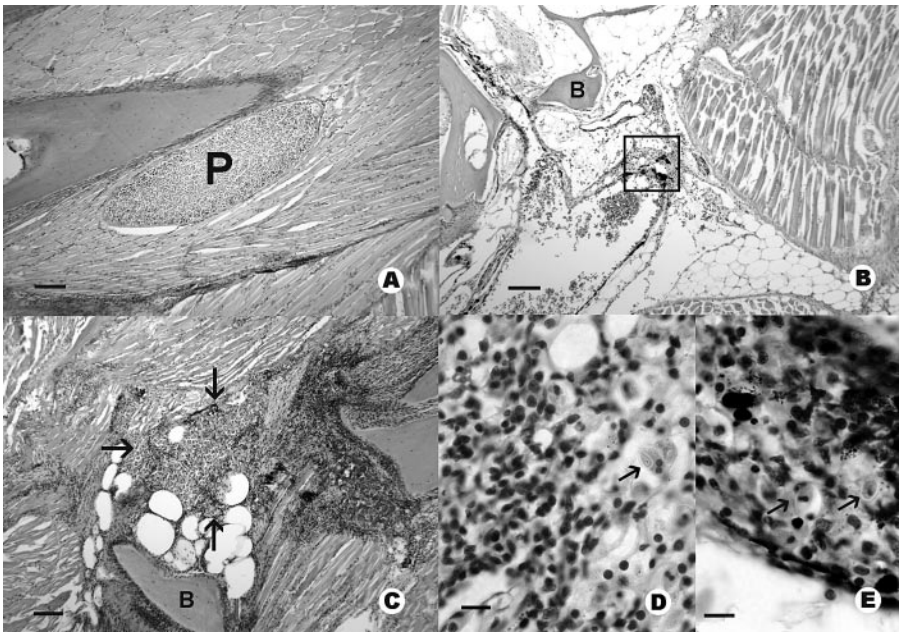


FIGURE 3.—*Myxobolus* sp. in histological sections of a northern pikeminnow with vertebral deformities. Panel **(A)** shows an intact plasmodium (P) next to a dorsal vertebral spine; bar = 100 μm. Panels **(B, C)** are low magnifications showing inflammatory lesions associated with vertebral deformities; bars = 100 μm. The box in (B) indicates a region shown in high magnification in (D); the letter B designates bone. In (C), arrows demark a lesion comprising chronic inflammation and free spores near a vertebra. Panels **(D, E)** are high magnifications showing spores (arrows) in chronic inflammation; bar = 10 μm.

performed a phylogenetic analysis with other closely related myxozoan sequences available from GenBank (Figure 7). Parsimony analysis yielded two equally parsimonious trees, the only difference between trees being placement of the group containing *M. cyprini* and *M. musculi*. One tree placed these as sister to all *M. pseudodispar* representatives, whereas the other tree placed them as sister to *M. pseudodispar* from *Scardinius erythrophthalmus* and *Rutilus rutilus*. In any case, the hypothesis that the *Myxobolus* species from northern pikeminnow is an outlier to all of these species has strong bootstrap support (Figure 7).

## Discussion

The high prevalence of vertebral deformities seen in fishes from the Willamette River near Portland, Oregon, has for many years been suspected of being caused by an unknown contaminant, but studies have not revealed a suspect contaminant in affected fish (L. Curtis, personal communication). Using multiple diagnostic methods, our survey of fish collected from all sites suggested that most of these deformities were caused by parasites. The association with metacercariae is most compelling, both diagnostically and epidemiologically. Examination of cleared fish added to our understanding of the metacercariae association and pathogenesis of the deformities. This method demonstrated a stronger association of the metacercariae with the deformities than was seen by other methods, probably because we were able to visualize entire metacercariae and vertebral deformities in

TABLE 3.—Cross-tabulation for presence (positive) or absence (negative) of metacercariae and *Myxobolus* in 63 histologically verified lesions in chiselmouths, northern pikeminnow, and peamouths from Ash and Kiger islands. Numbers in parentheses are percentages. A chi-square test rejected the hypothesis of the independence of rows and columns ($P = 0.0001$).

| | *Myxobolus* negative | *Myxobolus* positive | Total |
|---|---|---|---|
| Metacercariae negative | 8 (12.7) | 21 (33.3) | 29 (46.0) |
| Metacercariae positive | 32 (50.8) | 2 (3.2) | 34 (54.0) |
| Total | 40 (63.5) | 23 (36.5) | 63 |

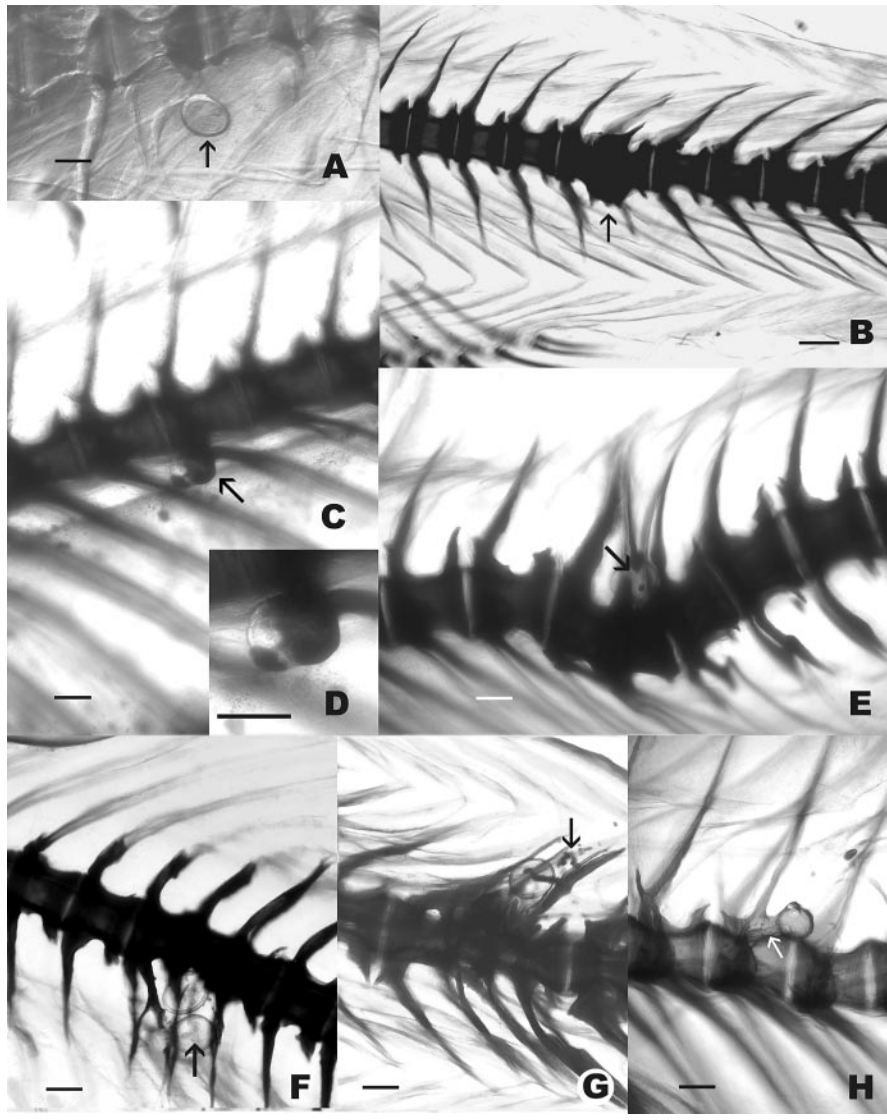


FIGURE 4.—Metacercariae associated with vertebral deformities in cleared northern pikeminnow (A–D) and chiselmouths (E–G); bars = 200 μm. Individual panels are as follows: **(A)** a metacercaria (arrow) at a site of missing hemal spine; **(B)** a dense vertebra (arrow), possibly associated with an internal parasite (see Figure 1A); **(C)** a bony proliferation (arrow) partially encasing a metacercaria at the base of a hemal spine; **(D)** a high magnification of (C); **(E)** a bent vertebral column with an associated metacercaria (arrow); **(F)** dystrophic hemal spines associated with two metacercariae (arrow); **(G)** additional neural spines and multiple dense concretions (arrows); and **(H)** a metacercaria cyst encased in bony material at the base of a neural spine (note the stalklike structure (arrow) connecting the cyst to the vertebra and the pores in cyst).

the same preparation. Cleared preparations revealed that many metacercariae were located at or very near to deformities. Most deformities without metacercariae were characterized by increased density and fusion of vertebrae, as visualized by using the clearing method (Figure 4). Metacercariae or their degenerated remains may have gone undetected within these structures by this method, but metacercariae were sometimes detected deep within vertebrae by histology (Figure 1A). Several metacercariae were associated with mineralized dropletlike structures in the surrounding muscle, most likely indicating dystrophic mineralization of foci of granulomatous inflammation. Direct cor-

TABLE 4.—Association of metacercariae with vertebral deformities in northern pikeminnow (NPM) and chiselmouths (CHM) from two affected sites—the Ash Island, Newberg Pool (Ash) and Wheatland Ferry (Wheat) regions—and the reference site, Kiger Island in the Willamette River. The analysis is based on microscopic examination of whole cleared fish stained with alcian blue and alizarin red S.

| Species | Lesion | Size (cm)[a] | No. | Location |
|---|---|---|---|---|
| CHM | Positive | 3.3 (1.9–3.8) | 25 | Ash, Wheat |
| CHM | Negative | 3.2 (2.2–3.8) | 13 | Ash, Wheat |
| CHM | Combined | 3.1 (2.6–3.8) | 38 | Ash, Wheat |
| CHM | Positive | 3.5 (3.2–3.8) | 11 | Ash |
| CHM | Negative | 3.5 (3.2–3.8) | 6 | Ash |
| CHM | Combined | 3.5 (3.2–3.8) | 16 | Ash |
| CHM | Positive | 2.7 (1.9–3.4) | 13 | Wheat |
| CHM | Negative | 2.6 (2.2–2.8) | 7 | Wheat |
| CHM | Combined | 2.7 (1.9–3.4) | 20 | Wheat |
| CHM | Positive | 2.2 (1.9–2.4) | 3 | Kiger |
| CHM | Negative | 2.2 (1.9–2.4) | 11 | Kiger |
| CHM | Combined | 2.2 (1.9–2.4) | 14 | Kiger |
| NPM | Positive | 1.9–4.5 (3.0) | 13 | Ash |
| NPM | Negative | 2.0–4.5 (2.7) | 24 | Ash |
| NPM | Combined | 2.8 (1.9–4.5) | 37 | Ash |
| NPM | Positive | 1.7 (1.7) | 2 | Kiger |
| NPM | Negative | 2.2 (1.8–2.7) | 14 | Kiger |
| NPM | Combined | 2.2 (1.7–2.7) | 16 | Kiger |

[a] Mean, with range in parentheses.
[b] 1 of 2.
[c] 1 of 16.

relation with abundance of metacercariae throughout the body (including fins) with number of vertebral deformities was not seen because a few fish had had heavy infections of the fins. Chiselmouths from the affected sites had about fivefold greater metacercaria abundance than did those from the reference site and threefold as many vertebral lesions. Retrospective examination of deformities seen in radiographs revealed dense regions (corresponding to regions of bone proliferation) and clear centers (corresponding to a parasite cyst; Figure 2). Such vertebrae often were fused and had additional spines, common deformities reported by Markle et al. (2002).

We identified two types of metacercariae in affected fish, a neascus-type and an *Apophallus* sp. Niemi and Macy (1974) described the life cycle of *A. donicus* from fishes collected in the Willamette River basin Oregon. Consistent with the present study, the second intermediate hosts in their study included northern pikeminnow as well as various other fishes. Moreover, they experimentally infected coho salmon (*Oncorhynchus kisutch*) and found that the metacercariae infected bony structures. They proposed that *A. venustus*, *A. similes*, and *A. brevis* were synonyms of *A. donicus* (Niemi and Macy 1974). Review of the taxonomy of the genus is warranted, but we conclude that the *Apophallus* sp. in the present study was likely *A. donicus* as described by Niemi and Macy (1974). These authors reported little host specificity for the metacercariae of *A. doncicus* in Oregon, which might explain why vertebral deformities have been found in many fish families (Markle et al. 2002). To our knowledge, this is the first report of *Apophallus* sp. in which the parasite elicited proliferation of existing bone. On the basis of histology and cleared fish findings, we conclude that some metacercariae actually penetrate vertebrae, whereas other metacercariae adjacent to vertebral structures induce proliferation of bony material around the parasite. In contrast, *A. brevis* metacercariae infect the somatic muscle of yellow perch *Perca flavescens* and cause a host response of ectopic bone (Sinclair 1972; Pike and Burt 1983; Taylor et al. 1994). Similar to *Apophallus* sp. in the present study, *A. brevis* in yellow perch apparently does not infect the visceral organs (Pike and Burt 1983). Taylor et al. (1994) described bony ossicles in yellow perch caused by *A. brevis*. One parasite we observed was completely encased in new bone yet exhibited a pore (Figure 4H) corresponding to the ossicle canal described for *A. brevis*. Metacercariae of heterophyid digeneans that infect the gills of freshwater fishes reportedly cause prominent dysplastic and proliferative changes in the gill cartilage (Blazer and Gratzek 1985; Olson and Pierce 1997). Infections by other metacercariae types have been linked to vertebral anomalies. Muscle infections by *Bucephalus po-*

TABLE 4.—Extended.

| Prevalence of vertebral deformities (%) | Infection prevalence (%) | Mean abundance | Vertebral deformities with metacercariae prevalence (%) | *Myxobolus* prevalence (%) |
|---|---|---|---|---|
| 100 | 100 | 6.0 | 95 | 0 |
| 0 | 85 | 2.6 | 0 | 0 |
| 64 | 94.5 | 4.6 | 95 | 0 |
| 100 | 100 | 5.5 | 91 | 0 |
| 0 | 100 | 3.3 | 0 | 0 |
| 67 | 100 | 5.0 | 91 | 0 |
| 100 | 100 | 4.6 | 100 | 0 |
| 0 | 71 | 3.3 | 0 | 0 |
| 66 | 90 | 4.4 | 100 | 0 |
| 100 | 100 | 2.3 | 100 | 0 |
| 0 | 27 | 0.5 | 0 | 0 |
| 21 | 43 | 0.9 | 100 | 0 |
| 100 | 77 | 1.2 | 54 | 0 |
| 0 | 29 | 0.4 | 0 | 19 |
| 35 | 38 | 0.6 | 54 | 19 |
| 100 | 50[b] | 1.0 | 50 | 0 |
| 0 | 0 | 0 | 0 | 0 |
| 12 | 6[c] | 0.125 | 50 | 0 |

*lymorphus* causes vertebral deformities in cyprinid fishes (Baturo 1980), and *Riberiora* sp. are suspected to be a major cause of supernumerary limbs and other vertebral changes seen in frogs in North America (Kaiser 1999).

Myxozoans, particularly *Myxobolus* spp., that infect bone are recognized causes of vertebral deformities. In addition to *M. cerebralis*, which affects salmonids (see reviews by Hedrick et al. 1998 and Bartholomew and Reno 2002), vertebral anomalies have been associated with *M. sandrae* infections of European perch *P. fluviatilis* in Scotland (Lom et al. 1991; Treasurer 1992), *M. ellipsoides* in European chub (*Leuciscus cephalus*) in England (Bucke and Andrews 1985), *M. cartilaginis* in centrarchid fishes in the United States

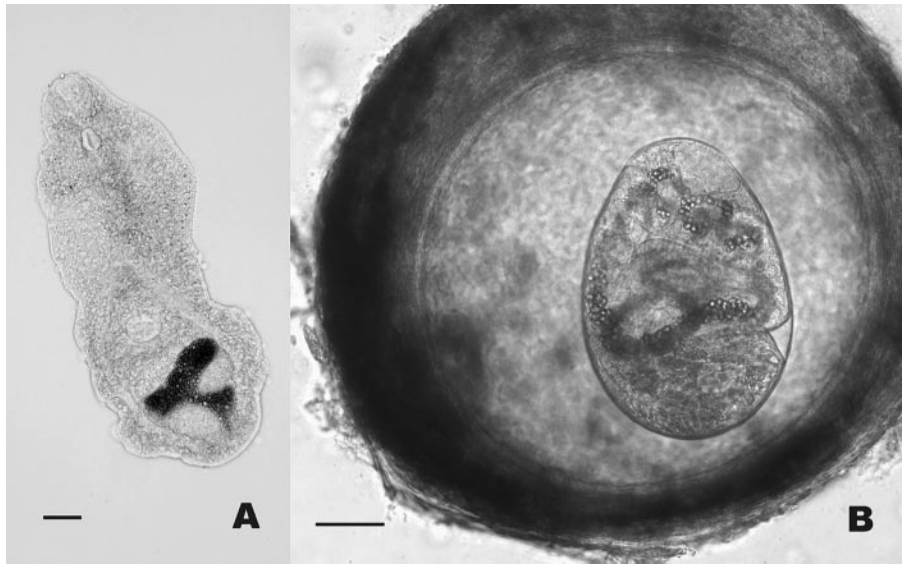


FIGURE 5.—Wet-mount preparations of metacercariae from northern pikeminnow; bars = 50 μm. Panel **(A)** shows an *Apophallus* sp., panel **(B)** a strigeid (neascus) with calcareous corpuscles (arrows) within a melanized cyst.

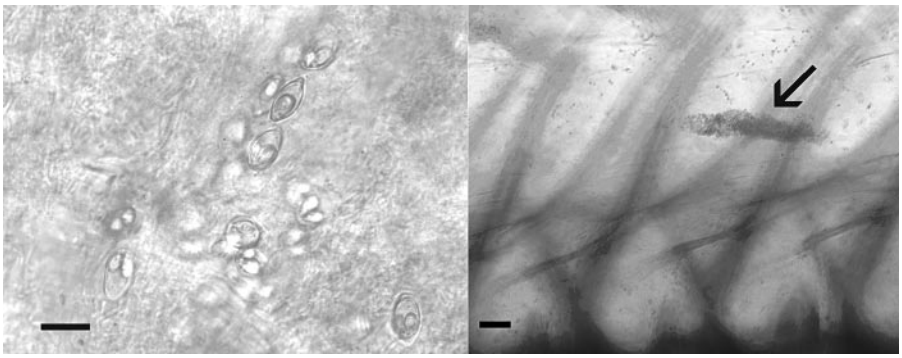

FIGURE 6.—Panel **(A)** shows spores of a *Myxobolus* sp. in wet-mount preparations (bar = 10 μm), panel **(B)** aggregates of spores (arrow) in somatic muscle of cleared northern pikeminnow (bar = 100 μm).

(Hoffman 1965), and *Triangula percae* in European perch in Australia (Langdon 1987).

The myxozoan in our study was most similar to *M. cyprini* or *M. pseudodispar* in spore morphology, site of development, and host. Spores of both infect the muscle of cyprinid fishes and are distinctly pleomorphic. Some authors have suggested that *M. pseudodispar* is a junior synonym of *M. cyprini* (Lom and Dyková 1992). Molnár et al. (2002) proposed that, based on rDNA analyses, *M. cyprini*, *M. pseudodispar*, and *M. musucli* should be maintained as separate species (although the first two are morphologically indistinguishable). Intact plasmodia do not cause significant tissue damage, but free spores of *M. cyprini* from ruptured plasmodia are disseminated to various organs, where they induce prominent inflammatory changes (Molnár and Kovács-Gayer 1985). Kent et al. (1996) were the first to report *M. cyprini* in North America. In contrast to previous reports, we observed free spores accumulated near vertebral structures and did not detect them in visceral organs. Moreover, our molecular analysis showed that the myxozoan was an outlier to the clade of closely related myxozoans formed by *M. cyprini*, *M. pseudodispar*, and *M. musculi*. Therefore, we cannot assign the parasite in this study to any of these species because it would render them paraphyletic.

The myxozoan that we observed was more common (as determined by histology) in northern pikeminnow than in chiselmouths and was not seen in peamouths. In contrast to the latter two fishes, vertebral lesions in northern pikeminnow appeared to be caused by both metacercariae and myxozoans. Based on cleared fish analysis, half of the deformities were associated with metacercariae (Table 4) and the other half with *Myxobolus* sp. (based

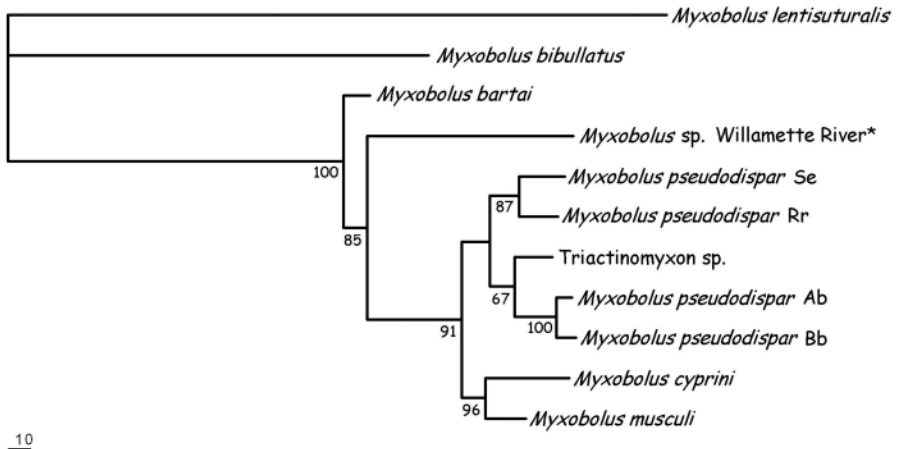


FIGURE 7.—Maximum parsimony dendrogram of *Myxobolus* sp. from northern pikeminnow. Bootstrap confidence intervals are shown at the nodes.

on histological analysis; Table 1). This pattern accounts for essentially all of the deformities in this species, given that the two parasites very seldom occurred together in the same lesion (Table 3). As determined by histology, *Myxobolus* sp. were more often seen in fish with vertebral lesions. In cleared fish, the myxozoan was seen only in northern pikeminnow from the affected region, but not in fish with deformities. Only large aggregates of spores could be seen in cleared fish. This is consistent with histological observations in that only individual spores within macrophages were seen in lesions; it is doubtful that we would have seen these spores in cleared fish.

In conclusion, the evidence presented here strongly indicates that parasites account for the high prevalence of vertebral deformities found in cyprinid fishes in the Willamette River in Oregon. More than 90% of the deformities seen in chiselmouths can be attributed to metacercariae (probably *A. donicus*). Combining observations from histology and cleared fish, we determined that *Myxobolus* sp. and metacercariae were equally responsible for the deformities seen in northern pikeminnow. Other questions remain, such as the apparent relationship of the infection and vertebral deformities with urbanized regions of the river. Increased trematode infections have been related to anthropogenic pollution and physical alterations of the aquatic environment by humans (Lardans and Dissous 1998). Kiesecker (2002) reported synergism between exposure to herbicides and pesticides and infections by metacercariae of *Ribeiroia* sp. and *Telochris* sp. as the cause of vertebral deformities in frogs. Although we have not detected contaminant levels that would be suspected of causing the lesions (L. Curtis, personal communication), perhaps an unknown agent in the affected region causes immunosuppression of cyprinid fishes in the river and thus predisposes them to infections. Our observation of an increased prevalence of two unrelated parasites in the affected region tends to support this hypothesis. It is also possible that factors not directly related to pollution, such as water flow dynamics or abundance of nonfish hosts involved in the life cycles of *Myxobolus* sp. and *Apophallus* sp., are important contributing factors.

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